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Synthesis and transport of chimaeric protein in Xenopus oocytes

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Synthesis and Transport of Chimaeric Proteins in Xenopus Oocytes

by

Kay Ellen Simon

DISSERTATION

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This thesis is dedicated to the two people who have made the greatest contribution to my education: my parents.

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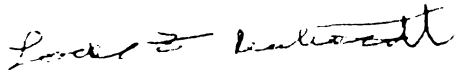
ABSTRACT

We have developed a simple and efficient method for obtaining high-level expression of cloned genes in Xenopus oocytes. This method involves the in vitro synthesis of capped transcripts followed by injection of total transcription products into oocytes. We have used this method to study protein secretion. We constructed a hybrid gene encoding a fusion protein (125E) composed of the signal sequence of beta-lactamse (a bacterial secretory protein) perfectly joined to the coding sequence for chimpanzee globin (a cytosolic protein). Our results show that when 125E is expressed in oocytes, the globin domain is translocated into the lumen of the endoplasmic reticulum (ER), and the signal sequence is cleaved. Pulse-chase experiments revealed that with time this globin domain is post-translationally modified, and that the modified form is rapidly and efficiently secreted into the oocyte medium. A second hybrid protein (GP) composed of the first 109 out of 143 amino acids of globin fused to the 5' terminus of preprolactin was also expressed in oocytes. Oocytes were capable of utilizing the internally localized signal sequence to translocate the prolactin domain. These results demonstrate the utility of the oocyte injection system for studying the various steps involved in the secretory pathway.



Chapter 1 Historical Introduction

HISTORICAL INTRODUCTION

The intracellular route for transport of secretory and membrane proteins in mammalian cells is well-established (1). Classical studies by Palade and colleagues utilized immunoelectron microscopy and subcellular fractionation techniques to approximate the route followed by newly synthesized proteins from their site of synthesis to their site of discharge in pancreatic exocrine cells. They showed that secretory proteins move from the rough endoplasmic reticulum (RER) through the Golgi complex, and that from the Golgi complex they travel via vesicles to the cell surface. Subsequent studies have attempted to identify important components of the transport machinery and to characterize the underlying mechanisms.

The initial events in secretion involve targetting of the nascent chain to the ER and co-translational translocation of the peptide chain across the ER membrane. The development of cell-free systems which faithfully reproduce the translocation event (2-5) set the stage for a more detailed biochemical analysis of these early events. Blobel and Dobberstein (2,3) formulated the "signal " hypothesis which proposed that the nascent secretory chain contains an amino terminal extension called the "signal sequence"; this sequence interacts with receptors in the ER

membrane to trigger formation of a ribosome-membrane junction and initiate translocation.

Two components which are required for the translocation event have recently been purified from canine pancreas (6-16); these are the signal recognition particle (SRP) and the SRP receptor, also called the docking protein. SRP is a complex of 6 polypeptides and a 7s RNA (6-10). SRP binds with high affinity to ribosomes that are synthesizing secretory proteins and causes arrest of chain growth after approximately 80 residues. The SRP-polysome complex then binds to SRP receptor which is an intergral protein in the ER, and this interaction results in release of SRP, renewed chain elongation, and extrusion of the polypeptide chain across the membrane into the ER lumen.

The mechanisms governing the subsequent journey of exported proteins remain obscure. It is generally believed that the intensive traffic in proteins and membranes is mediated by transport vesicles. A number of investigators have purified subpopulations of intracellular organelles with the hopes of identifying specific components critical to their transport function. Coated vesicles, which were first described by Roth and Porter (17), have been implicated as transport vehicles for both endocytotic and

exocytotic events. A widely used purification procedure was introduced by Pearse (18) who identified "clathrin" as the major coat protein. An improved purification scheme was presented by Pfeiffer and Kelly (19) who fractionated them further into subpopulations (20). More recently, Payne and Sheckman (22) have presented evidence which challenges the model that postulates coupling between the formation of coated vesicles and protein secretion. These authors constructed and characterized yeast strains deficient in synthesis of clathrin heavy chains and showed that these cells were able to secrete invertase at a nearly normal rate. Kelly and coworkers have also outlined procedures for obtaining highly purified preparations of secretory granules from the Att-20 pituitary cell line (23-24) and of synaptic vesicles from the Elasmobranch electric organ (25). More recently, Schroer et al have extended these studies by demonstrating that these purified synaptic vesicles retain their capacity to move when added back to extruded axoplasm or purified microtubules (26,27).

Reconstitution of transport events in cell-free extracts is an essential step towards understanding these complex cellular events. James Rothman and colleagues have reconstituted a different step in the intracellular pathway

(28-33). Rothman presented evidence that the VSV G glycoprotein is transported between successive compartments of the Golgi stack in a cell-free system. Rothman utilized mutants defective in various steps in glycosylation to design an assay that measures intercomparmental transfer. Golgi fractions were purified from a VSV-infected mutant cell line (missing a key enzyme, GlcNAC transferase) and from a wild type uninfected cell line. Mixed incubations of the two Golgi populations showed that the VSV protein which had been synthesized in donor mutant cells was glycosylated by the GlcNAC enzyme present in the Golgi stacks provided by the acceptor wild-type cells. Thus, transfers can occur between subcompartments in different stacks. Rothman has demonstrated that the cell-free system is highly specific, measuring only transport between sequential compartments in the Golgi stack.

Another approach to the transport issue has been to attempt to identify putative "sorting" signal, domains which direct the passage of transported proteins to the appropriate subcellular organelles. The most thoroughly characterized recognition system involves the segregation of newly syntehsized lysosomal enzymes. The asparagine-linked high mannose sugars of lysosomal enzymes are selectively modified in the Cis Golgi. The phosphate group

specifically binds to an intracellular receptor, and the receptor-ligand complex is selectively transported to the lysosome (34-40).

One of the more powerful techniques for identifying sorting signals involves in vitro modification of DNA coding for a protein, followed by transfection of the modified DNA into an appropriate cell. Experiments in which mutations were introduced into the clond DNA sequences coding for membrane proteins have led to the suggestion that sequences important for efficient transport may reside within either the transmembrane domain or the cytoplasmic tail (41-44). The two membrane proteins which have been the most extensively examined are the VSV G protein and the influenza virus hemagglutinin. It was found that mutations in the carboxy-terminal cytoplasmic domain frequently resulted in a decreased rate of protein transport between the endoplasmic reticulum and the Golgi apparatus without affecting subsequent transport to the cell surface. The type of alteration which most frequently causes major defects in transport involves the addition of novel nonhomologous amino acid sequences onto truncated or intact cytoplasmic tails. One interesting experiment involved an examination of the transport of a hybrid protein consisting

of the complete sequence of the rat growth hormone gene fused to the membrane spanning and cytosolic domains of the VSV G protein. This fusion protien was anchored in the microsomal membrane in the expected transmembrane configuration, was transported to the Golgi apparatus and was esterified to palmitic acid, but it was not transported to the cell surface. It was suggested that the sorting signal which allows rapid secretion of soluble rGH does not function when the protein is bound to the membrane, and that anchoring of rGH rendered it unable to interact with one or more receptors involved in transport within the Golgi or from the Golgi to the cell surface. The fact that this particular hybrid molecule was transported to the Golgi suggests that its inability to continue further in the transport pathway was not due to a lack of solubility.

Another method for introducing macromolecules into cells is by injection. In 1971, Gurdon and co-workers worked out the methodology for injection of RNA into oocytes (45,46). His results demonstrated that oocytes injected with mRNA purified from rabbit reticulocytes efficiently translated this RNA and produced large quantities of hemoglobin. Oocytes are very large cells (1-1.5mm diameter), and each oocyte has a synthetic capacity equivalent to that of 10-6 cells in culture. It is

therefore possible to perform a biochemical study on a single cell.

There have been numerous studies characterizing secretion from oocytes expressing various molecules which were synthesized from injected mRNA (47-54). These studies demonstrated that injected oocytes selectively export proteins which are secreted in their native environments; non-secretory proteins, such as rabbit hemoglobin are not exported. Subcellullar fractionation studies revealed that secretory proteins were sequestered in vesicles, whereas proteins which are normally cytosolic remained in the cytosolic fraction of injected oocytes. It was also found that different secretory molecules were secreted from oocytes at very different rates, specifically, lysozyme secretion from oocytes is 12 times that of ovalbumin (49).

The stage 6 Xenopus oocytes normally utilized in these studies are cells which are arrested at the prophase stage of meiosis. These oocytes have the appearance of a large, round cell with a very dark side (called the animal pole), a very light, unpigmented side (called the vegetal pole), and a white band surrounding the complete circumference and separating the two poles. Morphological studies (51,52) have shown that oocytes contain "cortical

granules" in close contact with the plasma membrane, and that these granules are capable of exocytosis. In a deeper region of the oocyte, smooth ER cisternae are intermingled with glycogen islets, pigment granules, yolk platelets, and mitochondria. The oocyte nucleus (germinal vesicle) is localized at the central region of the animal pole, and Golgi apparati are seen in both animal and vegetal poles, both in the cortical region and in deeper regions.

The oocyte has been reported to perform a number of modifications on exogenous proteins expressed in injected cells (for reviews see 53-54). Oocytes are capable of hydroxylating proline in collagen, and of N-acetylating the N-terminal methionine of aA2-crystallin. They have also been shown to assemble functional acetylcholine receptors which are transported to their appropriate location on the cell surface. Injections of mRNA purified from rat spleen cells showed that oocytes were capable of assembling IgG tetramers which were secreted into the medium. These tetramers contained their normal disulfide bonds, and were N-glycosylated. Though a number of exogenous proteins have been shown to receive the core oligosaccharide groups, the subsequent mannose trimming and replacement steps in oocytes differs from that normally observed in cultured cells. In both human chorionic gonadotrophin and rat

prostatic binding protein, mannose trimming is either absent or incomplete. Secreted IgG chains are still sensitive to Endo H, again suggesting that these chains have not been completely trimmed.

At present, little is known about the various export pathways which may exist in the oocyte, and which of the various modifications this cell-type is capable of performing. It was our intention to begin to characterize more fully the oocyte secretory pathways. We began our approach by developing a simple and efficient method for attaining high-level expression of cloned genes by injecting transcripts synthesized in vitro. Hybrid proteins containing putative sorting signals were then expressed and analyzed for their ability to be exported through the vesicular transport system. This work sets the stage for further analysis of the secretory pathway in Xenopus oocytes.

Chapter 2 Materials and Methods

MATERIALS and METHODS

Materials: All constructions used in this paper were placed in the pSP64 vector which was obtained from Promega Biotech. The constructions utilized in this paper were the generous gifts of Eve Perara and Vishu Lingappa and the details for their construction are outlined in ref. 87. The coding regions for the hybrid proteins encoded by these genes are shown in Figure 1. Plasmid G1E contains the entire coding region (143 amino acids) for chimpanzee a-globin. Plasmid pSP125E contains the complete signal sequence (22 amino acids) of b-lactamase perfectly fused to the entire coding region for chimpanzee globin. pSPBP3 contains the entire coding region for bovine preprolactin. pSPGP contains the entire coding region for bovine preprolactin inserted, in frame, 17 codons downstream from the initiation codon of chimpanzee a-globin. Hatched bars represent signal sequences, open bars represent coding regions from mature proteins, arrows point to the cleavage site at the 3' end of the signal sequence. Rabbit anti-human globin antisera was obtained from Cappel laboratories,Cochranville, PA. Rabbit anti-bovine prolactin was from United States Biochemical Corp., Cleveland, Ohio. All restriction endonucleases, SP6 RNA

polymerase, T4 DNA ligase, and Klenow fragment of E.Coli DNA polymerase were from Boehringer Mannheim Diagnostics Inc., Houston , TX, or from New England Biolabs, Beverly, MA. RNase inhibitor was from Promega Biotec, Madison, WI. Trypsin, Traoslyl, and Proteinase K were from Boehringer Mannheim Diagnostics, Inc., Piscataway, NJ. Protein A-Sepharose was from Pharmacia Fine Chemicals, Piscataway, NJ. Xenopus Laevis frogs were obtained from Nasco.

Microinjection of Xenopus oocytes: Xenopus oocytes were manually dissected, and were subsequently injected and labelled in modified Barth's solution containing S-35 MET as described (45).

In Vitro Synthesis of Capped Trancscripts: Purified plasmids were linearized with PVU11, extracted in phenol/chloroform, ethanol precipitated, and resuspended in water. Transcription was carried out in 10ul volumes containing 1.25 ug linearized DNA, 2U SP6 polymerase, .25 mg/ml calf liver tRNA, .5mMATP, .5mMCTP, .5mMUTP, .1mMGTP, .5mMGpppG (caps), .2MDTT, .2mM spermidine, 6mM MgC12, 40mM Tris-C1, pH7.5, and supplemented with human placental RNase inhibitor. Reactions were carried out at 40C for 1 hr and

aliquots used directly for injection or translation.
In Vitro Translation: 4ul aliquots of total transcription products were added to translation reactions of 20ul volume containing 6.5 ul of wheat germ extract, 20 uCi 35S-methionine, 40uM each of the other 19 amino acids - metnionine, 20mM Hepes, pH 7.5, 80 uM spermine, 1mMDTT, 1mM GTP, 1mM ATP, 6mM creatine phosphate, 0.4mg/ml creatine phosphokinase, 0.1mg/ml calf-liver tRNA with final concentrations of magnesium acetate and potassium acetate adjusted to 2mM and 150 mM respectively. Reaction mixtures were carried incubated at 24C for 1.5 hrs in the absence or presence of 2U/ml dog pancreas rough microsomes (prepared as described, reference 2).

Immunoprecipitation of Oocyte translation products: Medium and oocytes were separated, and the medium was then suspended in 4x volume Buffer A (1% Triton x-100, 0.1M Tris, pH7.5, .1M NaCl, 0.01M EDTA, 1mM PMSF; oocytes were homogenized in 20ul/oocyte of Buffer A. 2ul antisera was added to each sample, and samples were incubated overnight at 4C. Following incubation, samples were spun at at 10,000g to remove aggregates, and the supernatants were then transferred to a fresh tube containing 15ul of a 50% slurry of protein A-Sepharose in Buffer A. Samples were

incubated for 1 hr. at 4C, washed 3 times with Buffer A and twice subsequently with 0.1M Tris, pH7.5 and 0.1M NaCl to remove residual Triton.

Pulse-Chase: Oocytes were injected with in vitro synthesized transcripts, preincubated for 5 hrs. and pulse-labelled for 1 hour in MBSH containing 5 mC/ml S35-met. Hot medium was then removed. oocytes were briefly washed in cold MBSH, and then incubated for varying lengths of time in MBSH containing 10% FCS and 20mM cold methionine.

Preparation of oocyte vesicles: Freshly labelled oocytes were homogenized in 40ul/oocyte cold 10% (w/v) sucrose, 150mM NaCl, 10mM MgAc, 20mM Tris buffer, pH7.6. 500 ul total homogenate was layered over a 1 ml cushion of 20% sucrose (w/v), 50mM NaCl, 10mM MgAc, 20mM Tris-Cl, pH 7.5, and spun at 15,000g for 1/2 hr.in the ultracentrifuge. The top 500 ul (cytosolic components) was removed, the rest of the supernatant aspirated, and the pellet (vesicles) resuspended in Buffer A.

Proteolysis of oocyte vesicles; freshly labelled oocytes were homogenized with a Dounce homogenizer 40ul/oocyte in 10% (w/v) sucrose, 50 mM NaCl, 10mM MgAc, 20mM Tris-Cl, pH

7.6. Each sample was divided into 3 aliquots: the first aliquot was a control with no added protease, and the other two aliquots were treated with .3mg/ml Proteinase K in the presence or absence of 1% Triton for 3 hours at 4C. Proteinase digestion was stopped by the addition of 2mM PMSF followed by boiling in 5x volume 1% SDS in 0.1M Tris-Cl, pH 7.5 for 15 minutes. Samples were then diluted 20-fold in a solution of 1% Triton, 0.1M Tris, pH 8.0, 10mM EDTA, 100 mM NaCl, and subjected to immunoprecipitation followed by gel electrophoresis.

fig.1

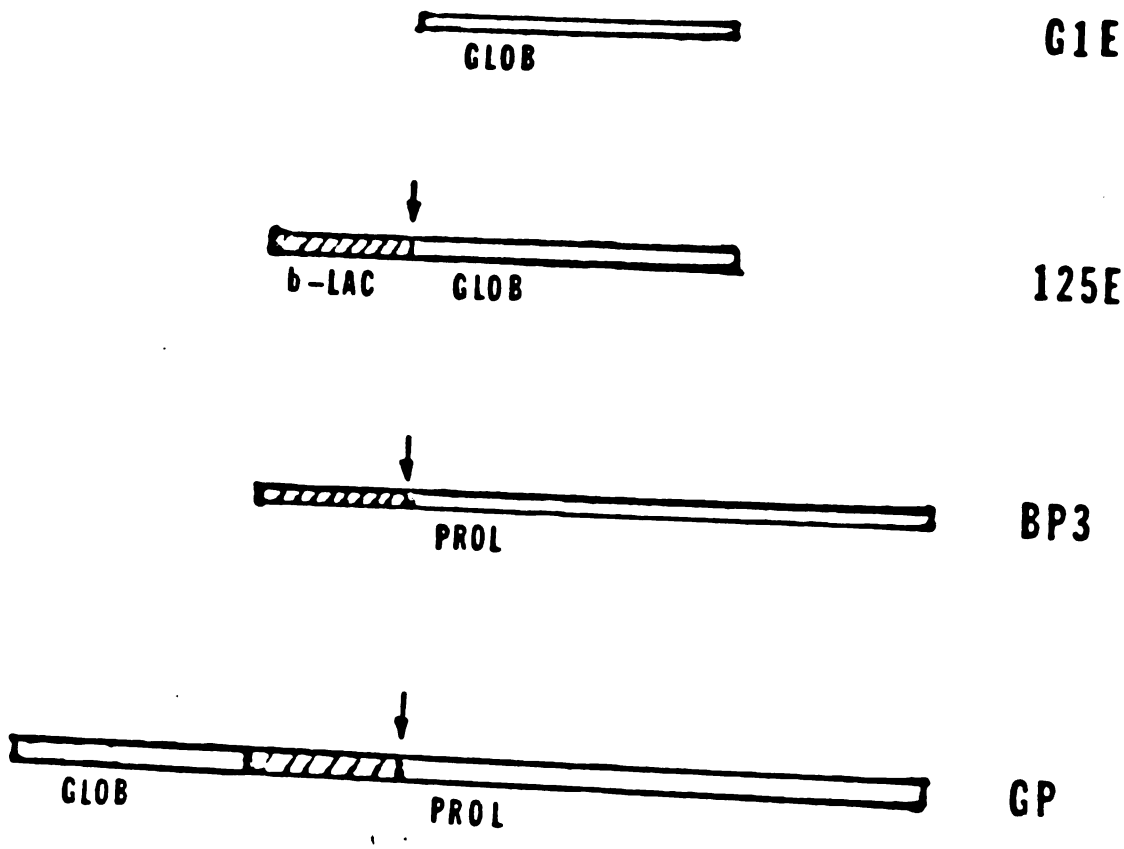


fig 1. Line diagrams of Reading Frames in SP6 Constructions

All genes were placed behind the 5' untranslated region of globin which had been placed upstream from the SP6 promoter. G1E contains the complete coding sequence for chimpanzee globin. 125E encodes a fusion protein in which the signal sequence of b-lactamase (the first 22 amino acids) has been perfectly joined to the complete coding sequence for chimpanzee globin. BP3 contains the complete coding sequence for preprolactin. GP encodes the first 109 (out of 143) amino acids of chimpanzee globin fused to the initial methionine of preprolactin.

INTRODUCTION

The ability of the Xenopus oocyte to efficiently translate injected mRNA is well established (45-50). In the past, material to be injected was obtained by purification of poly A+ RNA from various tissues or cultured cells. The use of purified RNA had a number of limitations: isolation of RNA from many tissues (especially those of highly differentiated cells) could be time-consuming and might also yield relatively low amounts of RNA, the mRNA isolated contained a heterogeneous population of transcripts representing all of the transcripts produced by the cells from which they were isolated, and the particular RNA of interest might represent a very small percentage of the total transcripts. Recent advances in recombinant DNA technology have made it possible to clone eukaryotic cellular genes, and to modify them in vitro. If one could obtain expression in oocytes of transcripts stnthesized in vitro from cloned genes, one would have the advantage of being able to introduce a highly pure preparation which, theoretically, could yield high levels of expression sufficient to perform a biochemical study on a single cell.

As early as 1975, Roberts et al (55) reported the development of an in vitro transcription system which

produced RNA capable of being translated in a cell-free system supplemented with wheat germ extracts. In this study, E. Coli RNA polymerase was utilized to transcribe an SV40 DNA template. Our laboratory was also able to produce translatable RNA in vitro from constructions containing the b-lactamase promoter and transcribed with the E.Coli RNA polymerase, however, the same RNA which translated efficiently in the cell-free wheat germ system, produced no detectable translation products when injected into oocytes. Transcripts prepared in vitro lacked a modification on the 5' end which, apparently, was required for efficient translation in vivo.

Most eukaryotic mRNAs are modified at their 5' termini with a "cap" structure consisting of 7-methylguanosine joined in a 5'-5' linkage to the penultimate nucleotide. It is believed that cap addition occurs during, or shortly after initiation of transcription. There is strong support for the idea that the 5' cap has a functional role in eukaryotic protein synthesis at the level of initiation. Several groups have reported that the presence of the 5' cap greatly effects the efficiency of translation in vitro (56-58). It has also been shown that the absence of a cap structure reduces the stability of RNA injected into oocytes and results in a

complete loss of detectable translation products (59, 60). For this reason, it seemed that addition of a 5' cap might be necessary for producing in vitro transcripts capable of being expressed after injection into Xenopus oocytes.

Recently, Melton et al (61-62) reported the construction of a set of cloning vectors which contain the bacteriophage Sp6 promoter upstream from a polylinker sequence . The SP6 system offers the advantage of utilizing a highly specific polymerase which is also very efficient.

In the following experiments, we outline a simple one-step procedure for synthesizing large quantities of capped transcripts in vitro. These transcripts are efficiently translated after injection into xenopus oocytes. Total transcription reaction mix can be injected into oocytes without the need for further purification, and all constructions so far examined were capable of being expressed.

RESULTS

Our initial characterization of the SP6 in vitro transcription system utilized the pSP125E fusion plasmid. This plasmid contains a hybrid gene encoding the b-lactamase signal perfectly fused to full-length chimpanzee globin and placed behind the SP6 promoter in the pSP64 vector. Plasmids were linearized at the PVU11 site and then transcribed in vitro in the presence or absence of .5mM GpppG caps. GpppG_ caps were chosen because the initial codon recognized by SP6 polymerase is a guanosine nucleotide. 125E transcripts were prepared in vitro and then translated in a cell-free system supplemented with wheat germ extract. Analysis of translation products by polyacrylamide gel electrophoresis in NaDodSO4 and subsequent autoradiography yielded two major products (Fig 2). The downward- pointing arrow refers to a protein which migrates to the position appropriate for 125E chains previously observed in linked transcription-translation systems. The lower molecular weight bands represents incomplete chains which most likely were translated from an internal initiation site produced from the initial methionine of the globin coding region which is retained at its 5' fusion junction. Both major bands could be immunoprecipitated with rabbit anti-human hemoglobin

antisera (data not shown).

Figure 2 demonstrates the effect of a 5' cap on the production of translatable RNA. Lane a shows the translation products from transcripts prepared in the presence of .5mM GTP and in the absence of caps. In lane b, transcripts were prepared in the presence of .1mMGTP and .5mM GpppG caps; and in lane c, transcripts were prepared in the presence of .1mM GTP and .5mM 7mGpppG caps. It is clear that the addition of GpppG caps to the transcription mixture had a dramatic effect on the production of translatable RNA; however, addition of the 7mGpppG caps had no effect. We asked if adding increasing amounts of polymerase resulted in the production of increasing amounts of translatable RNA. Lanes d, e, and f show translation products from transcripts produced in the absence of caps, in the presence of .5mM GpppG caps, and in the presence of .5mM 7mGpppG caps respectively, however, lanes d-f were transcribed in the presence of 1.2 units SP6 pol/10ul reaction polymerase, whereas lanes a-c were transcribed in the presence of .4 units/10ul. In lane g, transcription products were prepared in the presence of 2 units/10ul transcription mix supplemented with .1mM GTP and .5mM GpppG caps. Thus, adding increasing amounts of enzyme greatly

elevated the amount of translation products obtained in the linked transcription-translation system. Lane h is a control showing translation if 1mg/ml rabbit globin mRNA under the same condition as lane a.

We wished to know if the increase in translation products observed in the presence of caps was due to an increase in the actual amount of RNA synthesized, or due to an enhancement of the translational efficiency of in vitro capped transcripts as compared with uncapped transcripts. Therefore, transcripts were prepared in the presence and absence of caps, and the amount of RNA synthesized was determined by measuring incorporation of 3H-ATP. Table 1 shows the effects of cap addition on incorporation of 3H-ATP at 3 different concentrations of SP6 polymerase. Addition of GpppG caps had no effect on the amount of 3H-ATP incorporation, however, addition of 7mGpppG caps appeared to inhibit transcription moderately (by 25%). Addition of increasing amounts of polymerase produced increasing amounts of RNA, although higher concentrations of polymerase (2u/ul) produced seemingly disproportionate increases in RNA synthesized. The same set of total transcription mixtures were then translated in a the cell-free system, and the amount of translation products synthesized was assayed by measuring incorporation of S-35

MET into TCA-ppt cpm. Transcripts were prepared with 1.2 U SP6pol/10 ul reaction in the absence and presence of GpppG caps and then translated. Transcripts prepared in the absence of caps led to incorporation of 2,454 TCA ppt cpm/10ul reaction, whereas transcripts prepared in the presence of caps led to incorporation of 137,232 TCA ppt cpm/10ul reaction. 3H-ATP incorporation was similar (700cpm/10ul) in both instances. We conclude that addition of caps to the transcription reaction has enhanced the subsequent translational efficiency of these transcripts by as much as 50-fold.

Figure 3 shows a graph of 3H-ATP incorporation and S35-Met incorporation with increasing amounts of polymerase in the reaction. One can see that the amount of translation products synthesized increases linearly with the amount of SP6 polymerase added to the original transcription reaction. Measurements of total products synthesized does not take into account the percentage of transcripts produced which are full-length. However, the autoradiogram of translation products shown in figure 2 shows that the percentage of full-length translation products seen in the linked translation system did not change either with addition of caps to the reaction mix or

with changes in the concentration of polymerase added.

We then tested the ability of in-vitro-synthesized capped transcripts to direct the synthesis of translation products in xenopus oocytes. Oocytes were injected with 50nl/oocyte of total transcription mix and labelled for 24 hours in MBSH medium containing S-35 MET. Oocytes were then homogenized in 20ul/oocyte of Tris-CL buffer, pH 7.6 containing 1% triton, immunoprecipitated with rabbit anti-human hemoglobin antisera, and analyzed by gel electrophoresis and autoradiography. Figure 4 shows the results of the oocyte injection experiment. Lane a is the molecular weight standards. Lane b shows the translation products prepared in vitro. The downward pointing arrow represents the unprocessed precursor; the lower molecular-weight band in lane b migrates to the same position as processed globin produced in the presence of dog pancreas microsomes. Lanes c and d show the products from injected oocytes immunoprecipitated with immune and non-immune antisera respectively. Lanes e and f are products from uninjected oocytes immunoprecipitated with immune and non-immune antisera respectively. Thus, oocytes injected with in vitro transcripts specifically synthesize the translation products encoded. Oocytes injected with

represent
the RER of oocytes and from which the signal sequence has been cleaved; further evidence for this will be presented in chapter 4. The downward-pointing arrowhead shows a higher molecular-weight band not seen in the in vitro system and we believe that this product is the result of a post-translational modification; further evidence for this interpretation will be presented in chapter 5.

To test whether adding increasing amounts of transcripts effected the amount of translation products syntehsized in oocytes, oocytes were injected with total transcription mix prepared in the presence of different concentrations of SP6 polymerase. Figure 5 shows the results of this experiment. Oocytes were injected with transcription mix, labelled for 15 hours in the presence of 1mCi/ml S35-met, and then processed in groups of 3 oocytes each. Lane a shows the immunoprecipitable products from oocytes injected with transcripts prepared in the presence of 1.5ug of DNA and .4U polymerase per 10ul volume. Lanes

b,c, and d are the controls representing injected oocytes immunoprecipitated with non-immune serum and uninjected oocytes immunoprecipitated with immune and non-immune serum resepctively. Lane e shows the immunoprecipitable translation products from oocytes injected with transcription mix prepared in the presence of 1.5ug DNA and 2U SP6 pol per 10ul volume; It is clear that increasing the amount of enzyme added to the transcription mix, and thus increasing the amount of RNA synthesized, has greatly enhanced translation of injected material, at least in the concentration range presented. From our measurements of 3H-ATP incorporation (see Table 1), we estimate that the concentration of of RNA injected in these experiments was approximately 10ug/ml at the lower enzyme concentration and 100 ug/ml at the higher concentration used. Lanes f and g in Figure 4 show the translation products from oocytes injected wth transcripts prepared in the presence of 2U/10ul polymerase, but with concentrations of DNA at .75 ug and .5ug per 10 ul respectively, The difference in translation products seen in lanes e and g demonstrates that adding increasing amounts of DNA to the transcription mix can also enhance levels of expression in injected oocytes, though increasing the concentration of DNA much

beyond a concentration of .75ug/10ul seems to have little effect. These results are in agreement with those of Melton et al (61-62), who recently reported that the optimum DNA concentration for transcription using SP6 polymerase is 100mg/ml.

In all the experiments described so far, we used DNA linearized at the PVU11 site to prepare transcripts; this was because there is no transcription termination signal contained in either our cDNA coding regions, or within the SP6 plasmid itself. For our linked in vitro translation systems, we routinely use transcripts prepared with non-linearized DNA and we have always obtained very efficient expression of the appropriate proteins with these transcripts. We tested whether it was possible to obtain efficient expression in oocytes injected with transcripts prepared from non-linearized DNA. In vitro capped transcripts were prepared with non-linearized DNA and with DNA linearized with PVU11. These transcripts were then injected into oocytes, and oocytes were then assayed for their ability to express the SP125E transcripts. Figure 6 shows that control oocytes injected with transcripts prepared from linearized DNA (lane a) expressed the globin products always observed, whereas oocytes injected with transcripts prepared with non-linearized DNA (lane b)

showed no detectable translation products. We suspect that, due to their large size, transcripts produced from non-linearized DNA cannot diffuse through the oocyte cytoplasm to the appropriate location of the synthetic machinery, and thus, their products are poorly, or not at all, expressed.

Figure 6 also shows the results of an experiment in which we tested the stability of transcripts injected into oocytes by assaying for their ability to express translation products aftera brief incubation period. In lanes c and d, oocytes were labelled for 30 hours each, however, lane c shows the translation products from oocytes labelled immediately after injection, and lane d shows the products from oocytes preincubated for 12 hours before labelling. It is clear that transcripts are stable for at least 12 hours. More recently, we have preincubated injected oocytes for as long as 36 hours before beginning our labelling, and still attain very high levels of expression. In general, we inject oocytes the day before we begin experiments, and this allows us to eliminate damaged eggs.

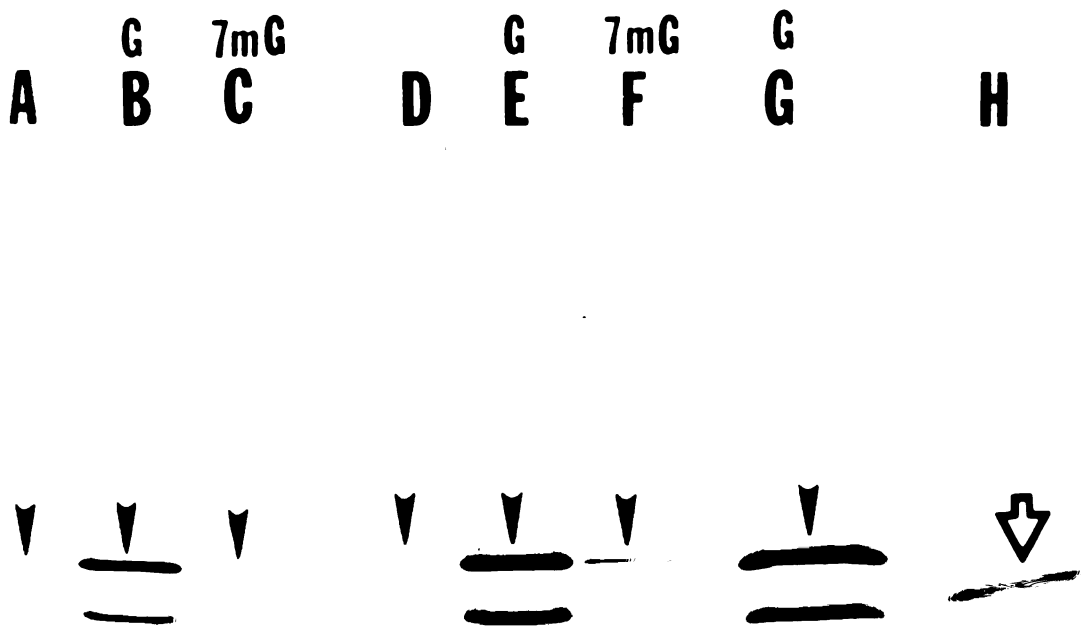

G
7mG
G
7mG
G
A
B
C
D
E
F
G
H

Fig. 2 Cell-free transcription-linked translation of pSP125E.

Transcription was carried out for 1 hour at 4C in the presence of.4U (A-C), 1.2U (D-F), or 2U (G) of Sp6 pol per 10ul reaction. All reactions included 1.25ug DNA linearzied at the PVU11 site, .25mg/ml calf liver tRNA, .2MDTT, 2mM spermidine, 6mMMgC12, 40mM Tris-Cl., pH7.5, 4U human placental RNAse inhibitor, .5mMATP, .5mMUTP, .5mMCTP. Lanes A,D, and H included .5mM GTP. Lanes B,C, and E included .1mM GTP and .5mM GpppG caps. Lanes C,F included .1mM GTP and .5mM 7mGpppG caps. Lane H is a control using 1mg/ml rabbit globin mRNA.

TABLE 1

SAMPLE	U POL/10ul	3H-ATP TCA-PPT CPM ABOVE BKGRND	S35-MET TCA-PPT CPM ABOVE BKGRND	UG RNA in 10 UL
TOTAL CPM		31,093	480,824	
BKGRND, NO POL	—	45	9,452	—
GTP	.4	174	—	.05
GpppG	.4	339	53,504	.11
7mGpppG	.4	219	—	.07
GTP	1.2	747	2,454	.24
GpppG	1.2	701	137,232	.22
7mGpppG	1.2	537	9,330	.18
GpppG	2.0	2,717	211,252	.86
RABBIT GLOBIN mRNA			106,332	10

fig.3

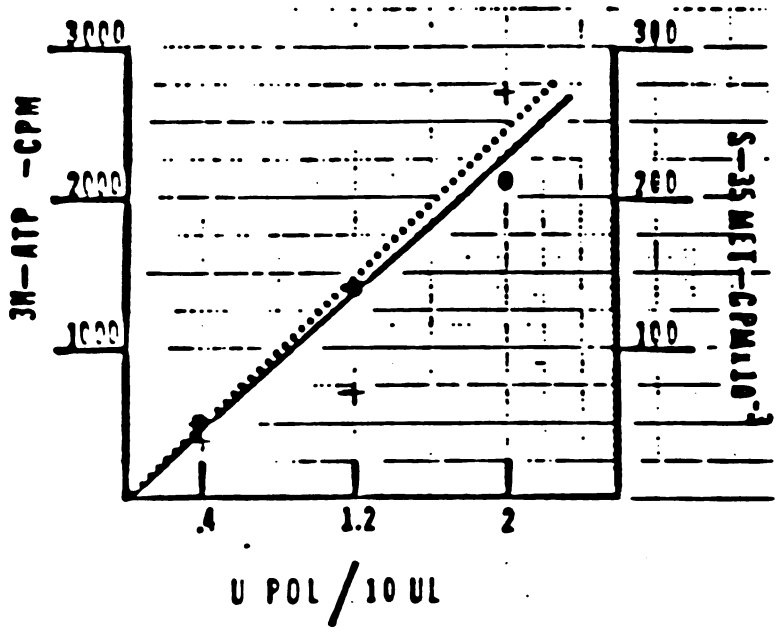

3000
2000
1000
300
200
100
3H-ATP -CPM
S-35 MET-CPM x10^3
.4
1.2
2
U POL / 10 UL

fig.3 Transcription-linked cell-free translation in the presence of increasing amounts of SP6 polymerase.

1.25 ug of linearized pSP125E DNA was transcribed in the presence of increasing amounts of SP6 polymerase. Solid dots and solid line represent TCA-ppt CPM produced in the linked translation system by incorporation of S35-MET. +s and broken line represent TCA-ppt CPM produced in the transcription system by incorporation of 3H-ATP.

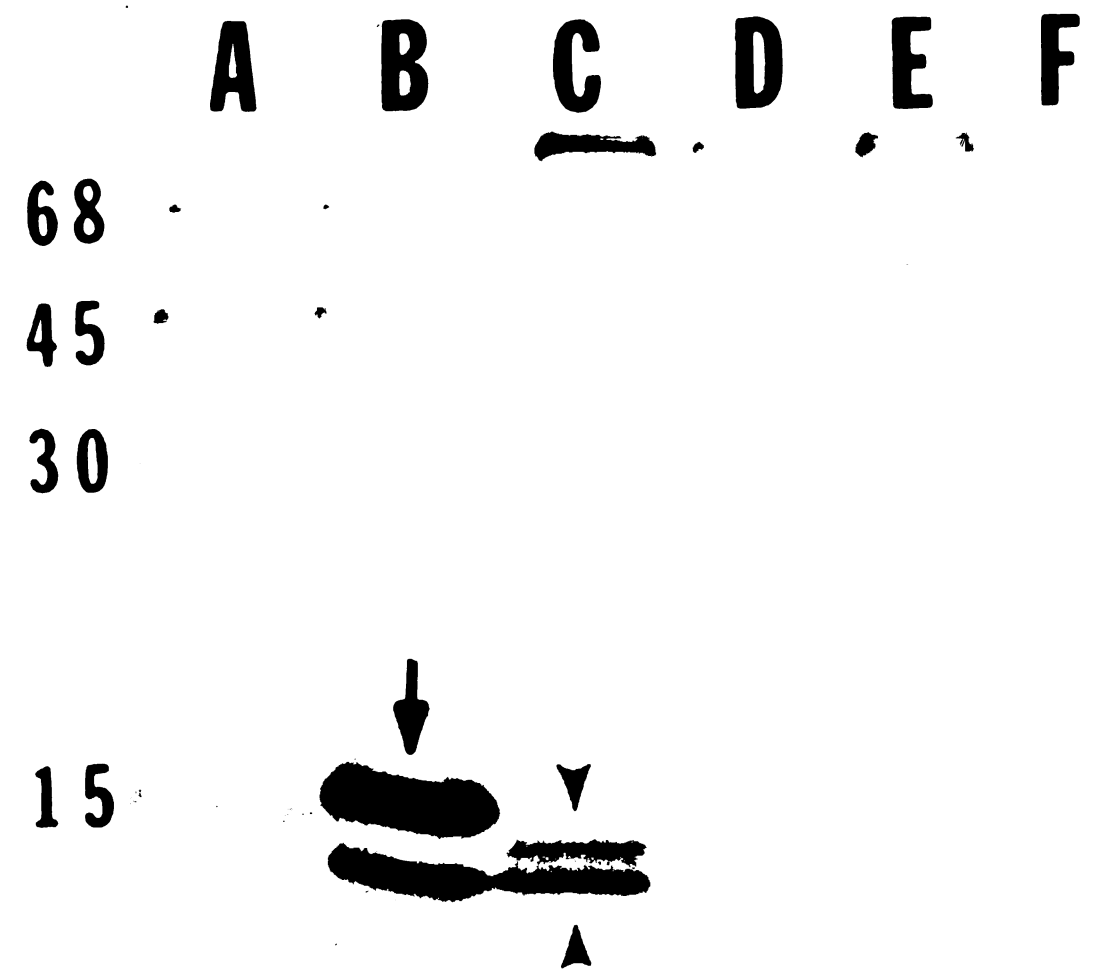

A B C D E F
68
45
30
15

fig.4 Expression of 125E in Xenopus oocytes injected with capped transcripts.

Capped mRNA was prepared in a cell-free reaction. Oocytes were injected with 50nl each of total translation products, and subsequently incubated for 24 hours in MBSH containing 1mC/ml of S35-MET. At the end of the incubation period, the medium was removed, and the labelled oocytes were homogenized in buffer containing 1% Triton and subjected to immunoprecipitation followed by gel electrophoresis and autoradiography. Lane A is the molecular weight standards. Lane B shows the products seen in the cell-free translation system. Lanes C and D are translation products from injected oocytes immunoprecipitated with immune and non-immune antiserum, respectively. Lanes E and F show the translation products from uninjected oocytes immunoprecipitated with immune and non-immune antiserum, respectively. Downward-pointing arrow refers to the unprocessed precursor observed only in the cell-free system. Upward-pointing arrowhead refers to processed globin chains. Downward-pointing arrowhead refers

to a processed globin chain of higher molecular-weight; this form is observed only in the oocytes.

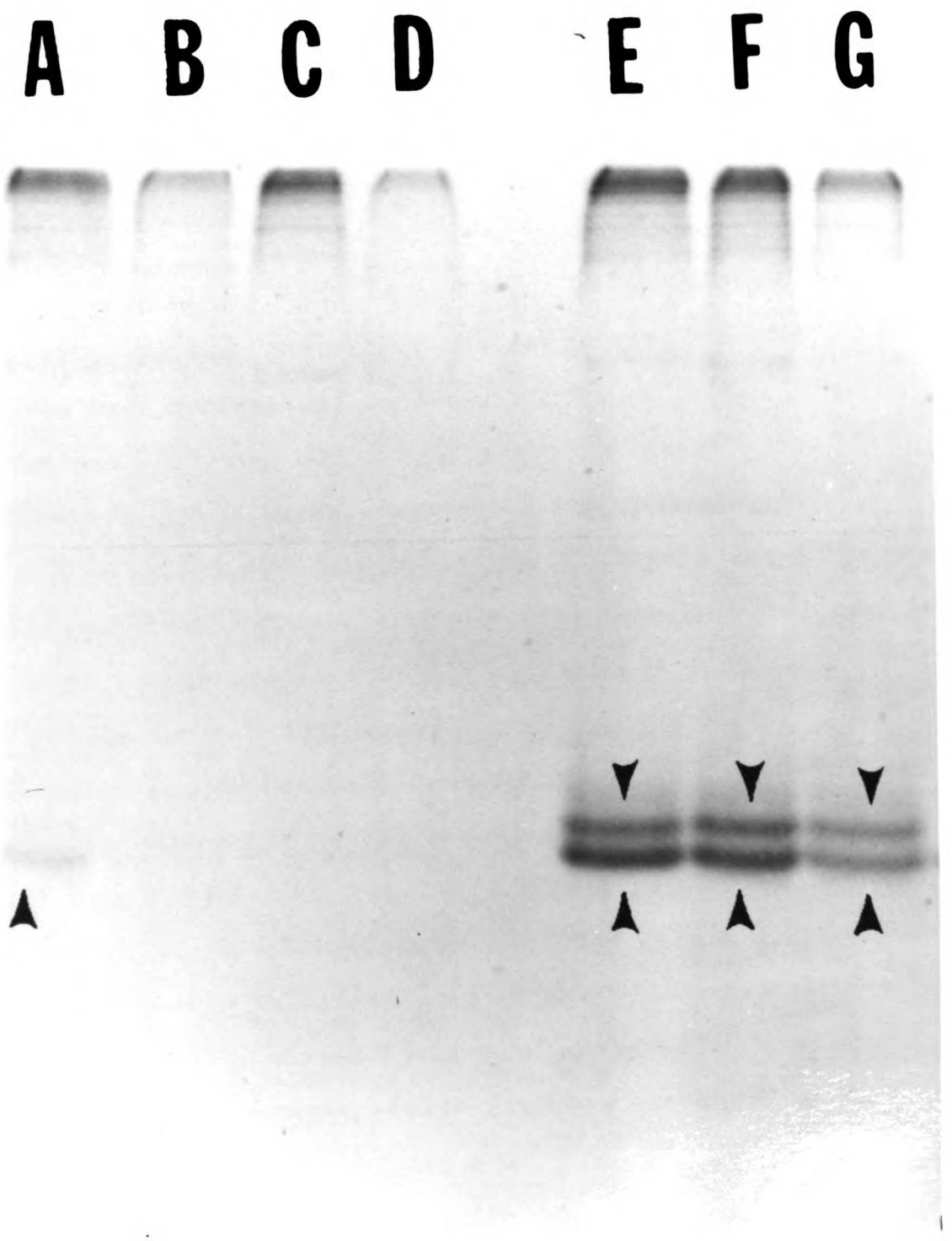

A B C D E F G

fig.5 Expression of SP125E capped transcripts in Xenopus oocytes; optimization of expression.

Capped transcripts were prepared in a cell-free reaction, and then injected into oocytes at volumes of 50nl/oocyte. Oocytes were labelled for 24 hours in 5ul/oocyte of MBSH medium containing 1mC/ml of S35-MET. At the end of the incubation period, the medium was collected, and the oocytes were homogenized in 20ul/ oocyte of buffer containing 1% Triton and subjected to immunoprecipitation followed by gel electrophoresis and autoradiography. Oocytes were processed in groups of 4. Lane A shows the the translation products obtained from capped transcripts prepared in the presence of 1.5 ug linearlaed DNA and .4U SP6 polymerase per 10ul transcription reaction. Lanes E-F show the products obtained from oocytes injected with transcripts prepared in the presence of 1.2 U polymerase and 1.5ug, .75 ug, and .5ug DNA per 10ul reaction, respectively. Upward-pointing arrowhead refers to processed globin which migrates to the same position as authentic globin. Downward-pointing arrowheads refer to higher molecular-weight globin products not observed in the cell-free system.

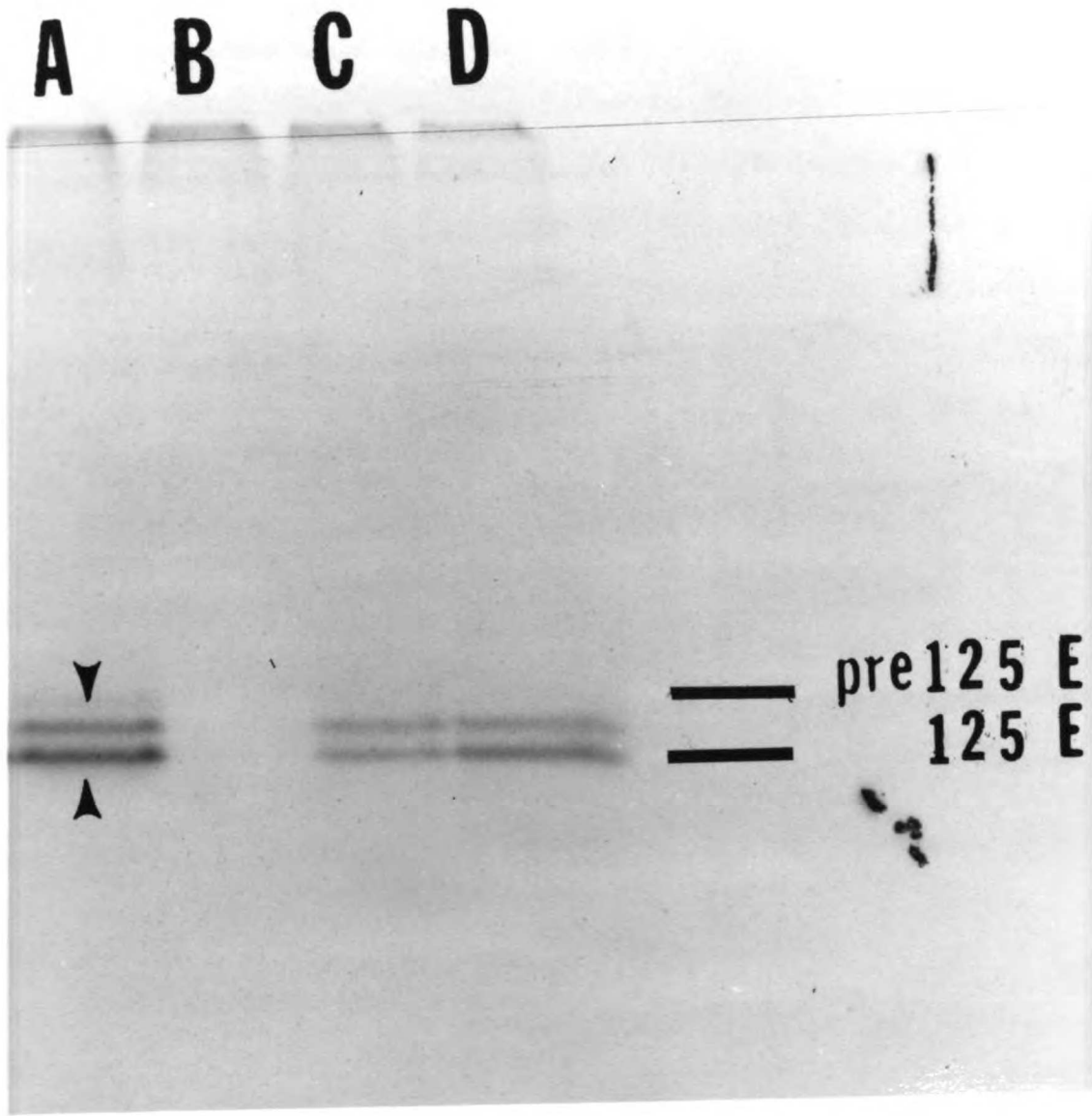

A
B
C
D
pre125 E
125 E

fig. 6 Effects of linearization and preincubation of RNA on expression of capped transcripts in oocytes.

Capped transcripts were prepared form pSP125E DNA which was either non-linearized (B), or linearized (A,C,D) with PVU11. Oocytes were then incubated in 5ul/oocyte of MBSH medium containing 1mC/ml of S35-MET for 24 hours (A-C), or, preincubated in unlabelled MBSH for 12 hours followed by incubation in labelled medium (D). Upward-pointing arrowhead refers to globin which comigrates with cell-free processed product; downward-pointing arrowhead refers to novel form not seen in cell-free system.

CONCLUSIONS

We have described a simple and efficient one-step procedure for the in vitro synthesis of capped transcripts which are efficiently translated after injection into Xenopus oocytes. Previous reports (59-62) demonstrated that a 5'cap enhanced translational efficiency in vitro and mRNA stability in Xenopus oocytes. Our results reconfirm the requirement of a 5' cap for expression of detectable quantities of translation products in oocytes injected with synthetically prepared transcripts.

We chose the SP6 promoter because of the ability of the companion enzyme to produce large quantities (microgram) of synthetic transcripts. The recent availability of the SP64 vector containing a polylinker with convenient restriction sites made this an even more attractive system. More recently, Doug Melton et al (61-62) have described the use of the SP6 vector/promoter system in some detail. These authors also obtained expression of translation products in oocytes injected with transcripts synthesized and capped in vitro. Melton et al prepared synthetic transcripts, purified the RNA by addition of DNAse followed by Sephadex G100 chromatography, and subsequently added 5' caps in an enzymatic reaction

utilizing guanylyltransferase purified from vaccinia virus. We have extended these studies by showing that the SP6 polymerase is capable of initiating transcription with the GpppG cap, and that capped transcripts can be prepared in a simple one-step reaction. Our results demonstrate that it is necessary to linearize SP6 plasmids prior to transcription in order to attain detectable quantities of translation products from injected oocytes.

We have also shown that after synthesis of capped transcripts, total reaction mix can be injected into oocytes, and high-level expression can be obtained. Injection of the total mix has no adverse effects on the viability of the oocytes, and we have maitained injected oocytes for as long as 100 hours after injection. In other studies (see chapters 4 and 5), we have shown that even after long-term incubation, there is no leakage of materials not normally secreted, whereas molecules normally intended for secretion are exported into the medium.

Total transcription mix can be stored at -70C for as long as 3 months without noticeable loss of activity, and we have frozen and thawed individual aliquots for as many as 4 times without any obvious loss of translational ability.

We have prepared a large number of constructions

which were expressed from synthetic transcripts prepared on the SP6 promoter. All RNAs capable of being translated in vitro were also expressed in oocytes injected with capped transcripts. Thus, this system provides a convenient method by which one can shuttle various proteins between the in vitro system and the in vivo system. Each oocyte has the synthetic capacity of 10-6 cells in culture, and, theoretically, injected RNA could represent as much as 10% of the total translated products. We can easily detect the products from one injected oocyte after 12 hours of fluorography. The ability to produce large amounts of functional transcripts, coupled with the large synthetic capacity of an individual oocyte, makes this a convenient system in which to study the in vivo behavior of newly-designed proteins.

Chapter 4 Translocation in Oocytes

INTRODUCTION

In eucaryotic cells, proteins intended for secretion are initially targetted to and translocated across the endoplasmic reticulum (ER) membrane. Most of these proteins are synthesized as precursors containing an amino terminal "signal" sequence which is proteolytically cleaved during, or shortly after, translocation (2-5). Via its binding to signal recognition particle (SRP), the signal sequence directs the formation of a ribosome-membrane complex, thereby initiating the translocation process (6-16). Little is known about the nature of the translocation machinery. One aspect of this problem concerns whehther a signal sequence which has been fused to a foreign amino acid sequence is capable of directing this "passenger" domain across the ER membrane, and if so, what are the constraints on this process.

In procaryotic cells, exported proteins contain a homologous signal sequence which is essential for directing these proteins across the inner membrane (63-69). Genetic studies in E. Coli suggested that fusion of a signal sequence to a previously cytosolic domain was not sufficient to specifiy export of the hybrid protein. A fusion protein consisting of the maltose binding protein

(an outter membrane protein) mino terminus joined to b-galactosidase (a cytosolic protein) was unable to be secreted into the periplasmic space. A fusion of the b-lactamase (a periplasmic protein) signal sequence perfectly joined to triose phosphate isomerase (a cytosolic protein) also failed to be secreted from E. Coli.

By contrast, Lingappa et al (70) demonstrated that a hybrid protein encoding this same b-lactamase signal sequence plus the first 5 amino acids of mature lactamase joined to chimopanzee globin could direct the transport of the globin domain into the lumen of microsomal vesicles in a cell-free system. It is not clear whether these seemingly discordant findings resulted from differences in choice of passenger domains, differences in the translocation mechanisms utilized by procaryotic vs. eucaryotic cells, or differences between the cell-free and in vivo systems.

In the present study, we have utilized 3 different hybrid genes in which the coding region for cytosolic globin was engineered behind, or, in front of, the coding region for a signal sequence. By microinjecting Xenopus oocytes with RNA synthesized in vitro, we were able to express these proteins and determine their fate in a eucaryotic cell. A combination of subcellular fractionation

and proteolysis of intact vesicles was used to assess the translocation competency of these "passenger" domains placed in different locations. Our results show that when fused to the carbosy side of a signal sequence, the previously cytosolic domain is efficiently translocated in a eucaryotic cell. Hybrid proteins in which portions of this same globin domain were fused to the amino terminus of preprolactin were also able to direct the translocation of globin domains, though with greatly reduced efficiency.

RESULTS

The results in this chapter are based on experiments which made use of two different constructions, the details of which are outlined in materials and methods. Plasmid pSP125E codes for a protein in which the entire signal sequence from beta-lactamase (22 amino acids) is perfectly fused to the entire coding region for chimpanzee globin. The pSPGP plasmid codes for a hybrid protein in which the first 109 amino acids at the amino termnius of chimpanzee globin (the complete coding region contains 143 amino acids) is fused to the initial methionine at the 5' end of preprolactin. Both constructions have been placed behind the SP6 promoter in the pSP64 vector.

We first tested the ability of the 125E translation products to be translocated by microsomal membrane vesicles in the cell-free system. Figure 7 shows the results of an experiment in which pSP125E templates were transcribed using SP6 polymerase, and mRNA was translated either in the wheat germ system alone (Lanes a and b) or supplemented with dog pancreas microsomal membranes (lanes c-f). Translation products were immunoprecipitated with rabbit anti-human globin antiserum (Lanes b-f) and analyzed by gel electrophoresis. Lane a is a control in which

translation products were immunoprecipitated with non-immune serum. Translation products synthesized in the absence of membranes (lane b) show a single band which migrates to the positiion appropriate for the unprocessed precursor (downward pointing arrow). When membranes were present during protein synthesis (lane c), a significant proportion of the nascent protein chains were shortened (proecessed) by a length consistent with the removal of the signal peptide. Protease digestion with trypsin post-translationally (lane d) showed that only the shortened chains were protected from protease digestion. Lane e demonstrates that the lower band is susceptible to protease digestion after addition of 1% triton which solubilizes the membranes. We thus conclude that 125E is capable of being translocated across the microsomal membrane, and that the signal sequence is cleaved. We currently do not know if the cleavage occurs at the appropriate site. Lane f shows the supernatant after sedimentation of membranes extracted with sodium bicarbonate, pH11.5, a procedure designed to strip off nonintegral proteins and content proteins from microsomal vesicles (7). Both the precursor and the cleaved product are found in the supernatant, and we can thus conclude that the processed globin molecule is present in a soluble form.

We asked how this same 125E translation product would behave in an in vivo situation. Capped transcripts were synthesized in vitro, and total transcription mix was injected into Xenopus oocytes in volumes of 50nl/oocyte. Injected oocytes were preincubated overnight and then labelled for 1-hour in 5ul/oocyte of MBSH medium containing 1mC/ml of S-35MET. Figure 8 demonstrates that a protein the size of authentic globin could be specifically immunoprecipitated with rabbit anti-human hemoglobin from oocytes injected with 125E transcripts. Lane a shows the translation products obtained from the cell-free system in the absence of membranes. The downward-pointing arrow refers to unprocessed 125E precursor, and the upward-pointing arrowhead refers to an incomplete chain which migrates to the same position as native globin. Lanes b and c show the translation products expressed by injected oocytes which were immunoprecipitated with immune and non-immune serum respectively. These oocytes have produced a single product immunoprecipitable with globin antiserum, and this protein migrates to the same position as native globin. Interestingly, we see none of the precursor normally observed in the in vitro system in the presence of microsomal membranes. Thus, this molecule must have been very efficiently translocated and processed. Uninjected

oocytes expressed no immunoprecipitable product (lane d). In chapter 3, our initial experiments showed that oocytes injected with 125E transcripts also expressed a second immunoprecipitable band which migrated at a slower rate. In these earlier experiments, injected oocytes were labelled for 24 hours, whereas in the present experiments, oocytes were labelled for 1-hour time periods. We believe that the higher molecular-weight band noted earlier represents a post-translational modification; evidence for this hypothesis will be presented in chapter 5.

In the case of 125E, we expressed a hybrid protein which contained a signal sequence placed at the amino terminus which is the natural location, and the product we obtained represented a protein which migrated to the position of native globin. We now asked if the oocytes were capable of utilizing a signal sequence in an internal position. Capped transcripts were prepared using the pSPGP plasmid and subsequently injected into Xenopus oocytes. Figure 9 shows the results from this experiment. Immunoprecipitation with anti-prolactin antiserum followed by electrophoresis and autoradiography produced two prominent bands (lane a). The upper band migrated to the position of the GP precursor normally observed in the cell-

free system in the absence of membranes, and the lower band migrated to the position of authentic prolactin. It appears that this particular protein has been processed, though relatively inefficiently. This was the first time that we observed expression of an uncleaved higher molecular-weight precursor after expression in Xenopus oocytes.

To determine whether the prolactin molecule had been translocated and cleaved, injected oocytes were homogenized in an iso-osmotic buffer containing .25M sucrose, and the suspension was then subjected to proteolysis with .1mg/ml trypsin for 3 hours. Lanes b and c show the results of protease digestion with trypsin in the absence and presence of 1% triton respectively. It is clear that all of the processed prolactin chains are protected, and thus they truly represent a set of translocated chains. However, none of the precursor chains are protected, and we conclude that the precursor molecules represent molecules which were not translocated. Since a large number of the precursor chains were processed, we wished to know what had happened to the globin-signal fragment which was released in the process. Lanes d-f show immunoprecipitation of translation products using anti-globin antiserum. In lane d, immunoprecipitation of total products yielded a single band

migrating at the position of the precursor, however we detected two extremely faint bands migrating near the position of authentic globin; we suspect that the globin-signal fragment may have been rapidly degraded, possibly due to its attatchment to the signal sequence which is believed to be degraded subsequent to cleavage. Lanes h-j demonstrate that the processed band previously observed after expression with 125E is also protected from protease digestion. Lane h shows total products in the absence of protease, and lanes i and j show translation products after protease digestion in the absence and presence of 1% triton, respectively. We can thus conclude that the globin product in oocytes injected with 125E has also been properly translocated into the RER lumen.

The absence of translation products immunoprecipitable with globin antiserum in oocytes injected with GP transcripts suggeste that there might be another explanation for the presence of processed prolactin sequestered in oocyte vesicles. The construction retains the initial methionine in the preprolactin coding sequence, and it is possible that the prolactin chains we observ originated from a preprolactin molecule generated by translation initiated at an internal site. To test this

for this possibility, oocytes were injected with GP transcripts , and, after a 24 hour preincubation, they were subsequently injected a second time with S35-MET dissolved in .25M hydroxyleucine. Hydroxyleucine is a leucine analogue which becomes incorporated into the amino acid chain, and its presence prevents translocation. If the processed prolactin chains were generated from preprolactin, then, in the absence of translocation, most of the products which were previously observed as mature prolactin chains should now be converted to chains which migrate to the position of preprolactin. If the processed prolactin chains were generated from the full-length GP precursor, then, in the absence of translocation, most of the products which were previously observed as mature prolactin chains should now be converted to chains which migrate to the position of preprolactin. If the processed prolactin chains were generated from the full-length GP precursor, then, in the absence of translocation, prolactin chains should now be converted to chains which migrate to the position of the GP precursor. Figure 10 shows the results of this experiment. Lane a shows total oocyte translation products immunoprecipitated with anti-prolactin antisera. Lane e shows total oocyte translation products immunoprecipitated after labelling in the presence of

hydroxyleucine. It is clear that in the presence of hydroxyleucine, a very small number of products appear at the position to which preprolactin molecules migrate; the vast majority of the immunoprecipitable chains were converted to the precursor form. The small number of products which are represented by preprolactin and mature prolactin cannot account for the large percentage of total products which were processed to prolactin in lane a. Lane c shows an experiment in which we unsuccessfully attempted to convert all of the GP precursor chains to prolactin by pulse-labelling with an injection of S35-MET diluted into purified SRP. Lanes b, d, and f show the same experiments after immunoprecipitation with globin antisera. Again, the amount of globin chains observed are negligible. Part of the explanation for our poor detection of globin molecules may also be that this truncated version of the globin molecule binds with very low affinity to the antisera we are using. This same antisera may recognize the precursor more easily because it assumes a different conformation.

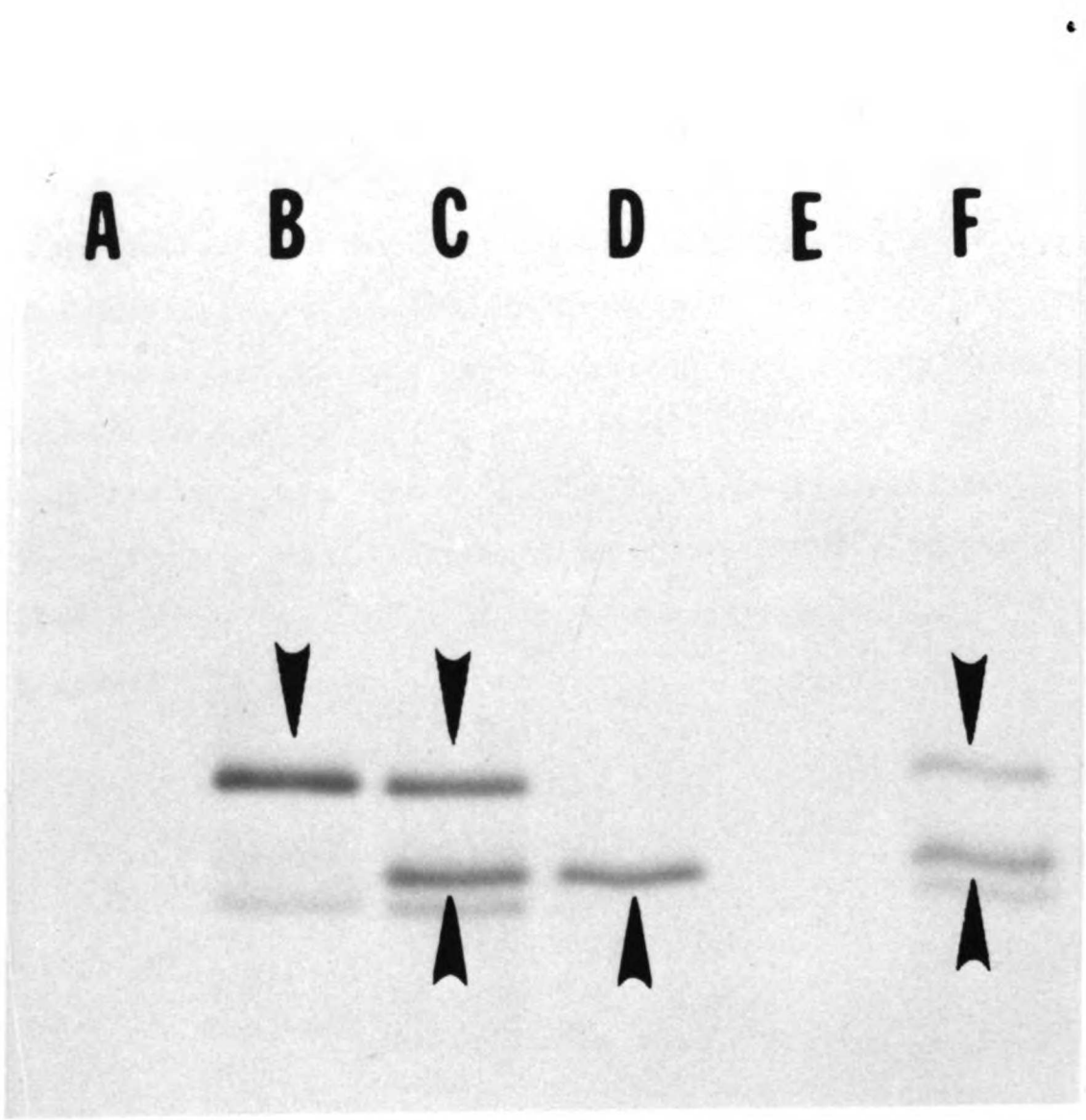

A
B
C
D
E
F

fig.7 Cell-free transcription-linked translocation-coupled translation of SP125E.

Transcripts were synthesized with SP6 polymerase and translation was in the wheat germ system alone (A,B), or supplemented with dog pancreas microsomal membranes (B,C,D,E) followed by post-translational assays. Aliquots of translation product were incubated with 0.1mg/ml trypsin (C,D,E) for 1 hour at 25C, then treated with 1mM PMSF to stop the reaction. In E, 0.5% Triton was included during proteolysis. Samples were immunoprecipitated with anti-globin antiserum (B-F), or non-immune serum (A) and prepared for SDS-PAGE.

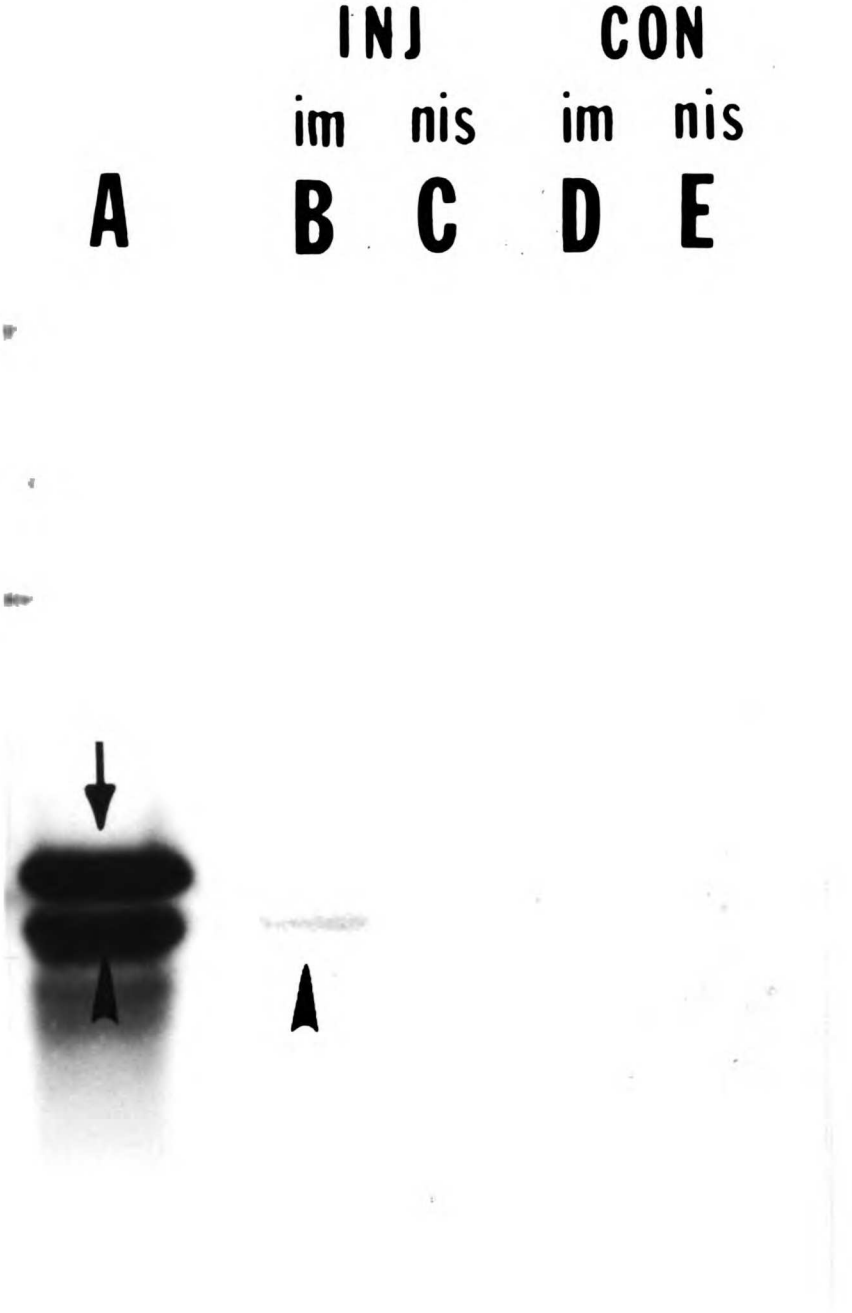

INJ
CON
im
nis
im
nis
A
B
C
D
E

fig.8 Expression of SP125E in oocytes injected with capped transcripts.

Capped transcripts were injected into Xenopus oocytes, and oocytes were preincubated for 24 hours in MBSH, followed by incubation for 1.5 hours in MBSH containing 5mC/ml of S35-MET. At the end of the incubation period, the medium was removed, and labelled oocytes were homogenized in 20ul/oocyte of buffer containing 1% Triton, subjected to immunoprecipitation, and analyzed by gel electrophoresis followed by autoradiography. Lane A shows translation products seen in the cell-free system. Lanes B and C show the products from injected oocytes immunoprecipitated with immune and non-immune antiserum, respectively. Lanes D and E show the products from uninjected oocytes immunoprecipitated with immune and non-immune antiserum respectively. Downward-pointing arrow refers to the precursor form observed only in the cell-free translation. Upward-pointing arrowhead refers to globin comigrating with cell-free processed product.

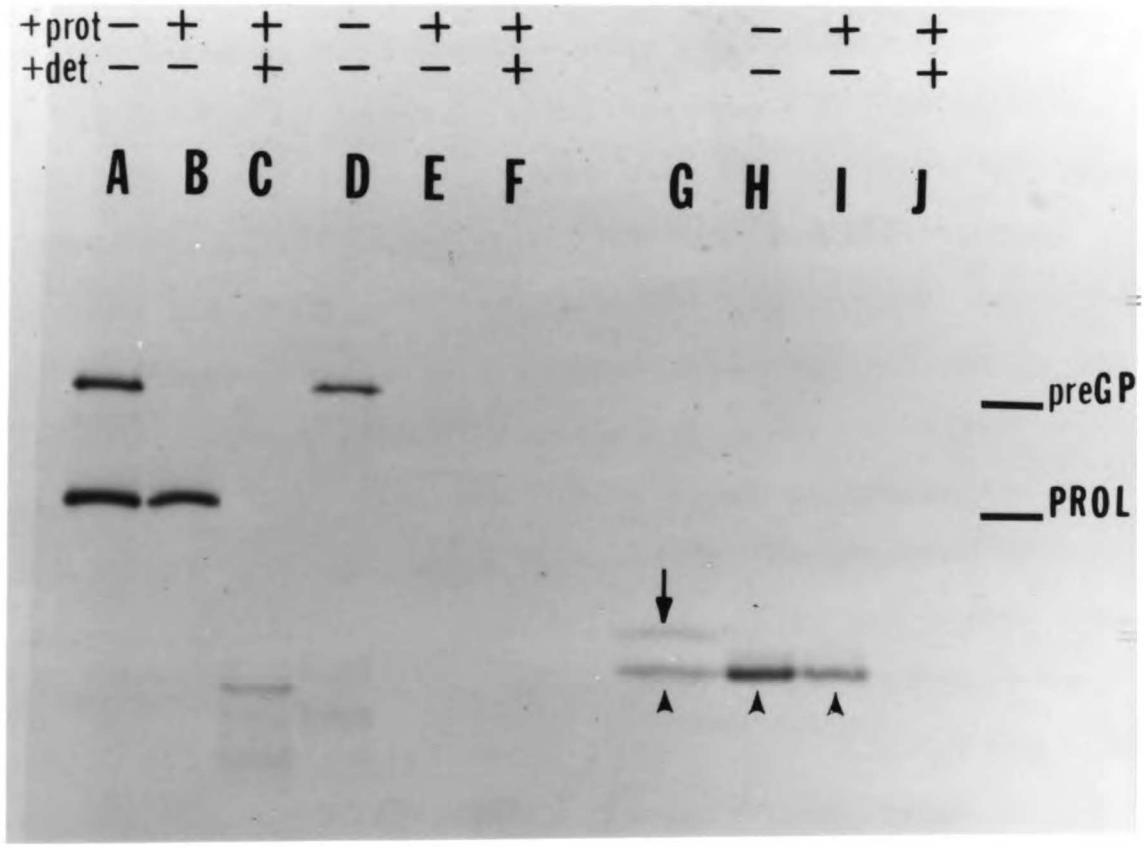

+prot − + + − + + − + +
+det − − + − − + − − +
A B C D E F G H I J
preGP
PROL

fig.9 Proteolysis of oocyte vesicles expressing GP and 125E.

Oocytes were injected with capped transcripts prepared with linearized SPGP DNA (A-F) and SP125E DNA (H-J), preincubated in MBSH for 24 hours, and subsequently labelled for 1.5 hours in the presence of 5mC/ml of S35-MET. Groups of 15 oocytes each were then homogenized in isoosmotic buffer containing 10mM MgAC and 10% sucrose, divided into aliquots, and sbuseqeutnly assayed. Lanes A-C were immunoprecipitated with anti-prolactin antiserum, and lanes D-J were immunoprecipitated with anti-globin antiserum. Lanes A,D, and H show total products; lanes B,E,I show total products digested in the presence of .1mg/ml trypsin and 10mM CaC12; lanes C,F, and J show total products digested in the presence of .1mg/ml trypsin and 10mM CaC12 with 1% Triton included. Lane G shows the 125E translation products seen in the cell-free system. Downward-pointing arrow refers to the precursor seen only in the cell-free system.. Upward-pointing arrowhead indicates the procssed chain of 125E.

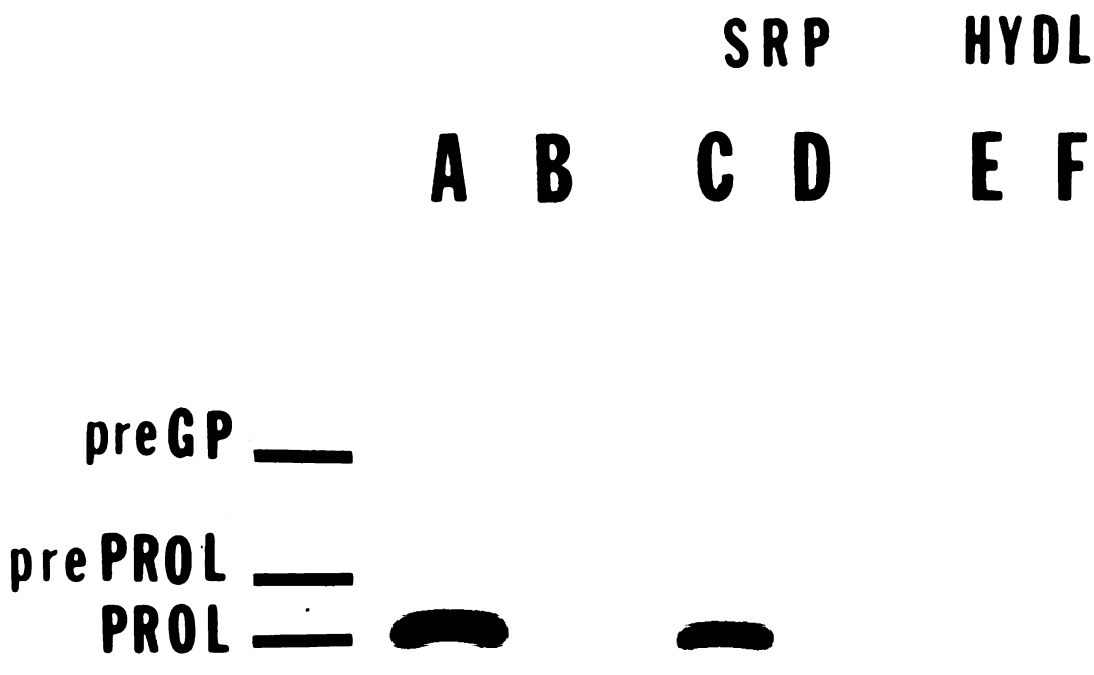

SRP
HYDL
A B C D E F
preGP
prePROL
PROL

fig.10 Expression of GP in oocytes subsequently injected with SRP and hydroxyleucine.

Oocytes were injected with capped transcripts prepared from linearized SPGP. After preincubation for 24 hours in MBSH, some oocytes were injected with H20 containing 100uC of S35-MET (lanes A-B), a second group was injected with 100uC S35-MET dissolved in 520ug/ml purified SRP, and a third group was injected with 25uC S35-MET dissolved in .25M hydroxyleucine . After the second set of injections, oocytes were incubated for 5 hours, and then homogenized and immunoprecipitated with either anti-prolactin antiserum (A,C,D) or anti-globin antiserum (B,D,F).

CONCLUSIONS

We have constructed hybrid genes coding for fusion proteins in which a previously cytosolic domain has been placed both upstream and downstream from a signal sequence. These fusion proteins were expressed in a cell-free system and in Xenopus oocytes, and were tested for their ability to direct the translocation of flanking domains. Previously, Lingappa et al (70) had utilized fusion proteins to demonstrate that the b-lactamase signal sequence plus 5 additional amino acids was sufficient to direct the translocation of the 142 amino acids coding for chimpanzee globin in a cell-free system. We have extended these studies to show that when globin is precisely joined to the carboxy terminal amino acid of the b-lactamase signal sequence, this domain is still capable of being translocated and processed in vitro. We have also shown that this same molecule is translocated and processed in vivo after expression in Xenopus oocytes. Earlier studies with bacterial systems had led to the suggestion that other information in addition to the signal sequence was required for translocation (67,68). One study showed that a fusion protein containing the lam B leader sequence

joined to b-galactosidase was not secreted across the plasma membrane, whereas a fusion product which contained virtually the entire lam B protein was efficiently secreted. In another study, a fusion of the b-lactamase leader sequence precisely joined to triose phosphate isomerase failed to be secreted from E. Coli. There are a number of possible explanations for the differences between these results and our own. One obvious difference lies in the choice of passenger domains. It is possible that transported domains must fufill certain (as yet unidentified) requirements to be compatible with the translocation apparatus, and that globin fulfills these requirements. Alternatively, it is possible that the translocation machinery of procaryotic and eucaryotic cells differ in some fundamental way; it is known that in bacteria, proteins grow to 80% of their final molecular weight before beginning translocation (69), whereas in the ER, translocation is tightly coupled to translation (2-3).

The present studies have allowed us to compare the behavior of fusion proteins in vivo and in cell-free systems. Our results show that the 125E fusion protein was efficiently translocated and processed in both systems. In contrast, the GP fusion protein behaves slightly differently in the 2 systems. Recent reports by

Perara and Lingappa (87) have shown that 20% of the globin-signal domains produced from the processed GP precursor are sequestered in microsomal vesicles in a cell-free translation system. In oocytes, we did not observe any translocated globin-signal domains, and these domains which remain associated with the signal sequence after cleavage appear to be rapidly and preferentially degraded. These results suggest that the current cell-free systems achieve a faithful, but only partial reconstitution of early biosynthetic events.

It is important to note that all of the bacterial studies utilized subcellullar fractionation techniques to determine the localization of fusion proteins. More recent studies used a hybrid protein encoding phoE (an outter membrane protein) fused to b-galactosidase to demonstrate that although subcellular fractionation suggested that this protein had been transported to the outter membrane, immunocytochemical studies revealed that the hybrid protein accumulated in the cytosol (71). Thus, data based on a single assay using only subcellular fractionation techniques must be interpreted with caution.

Expression of the pSPGP construction allowed us to ask a slightly different set of questions about how signal

sequences function in a eucaryotic system. We asked whether the translocation apparatus was capable of utilizing what was originally an amino-terminal signal when artificially poaced in an internal position. Our results show that the oocytes were capable of translocating the prolactin amino acid sequence on the carboxy side of the signal, and that the chain was processed. However a significant proportion of the total products were represented by uncleaved precursor, and none of the precursor was sequestered inside vesicles. We have expressed a large number of hybrid proteins in Xenopus oocytes, and in all other cases observed, there were no detectable precursor products. Thus, the oocyte used this internal signal, but less efficiently than other signals which maintained their natural position at the amino terminus. Interestingly, the signal-globin fragment was detected in very small quantities. We suspect that this part of the molecule was degraded due to its attatchment to the signal sequence which is most likely rapidly degraded after cleavage. An alternative explanation was that the oocytes had not utilized the internal signal, and that authentic prolactin had been generated from a preprolactin chain produced by initiation of translation at an internal site created by the continued presence of the preprolactin

initialy methionine at the fusion point. Our experiments in which we inhibited translocation with hydroxyleucine demonstrated that though there may have been a small amount of internal initiation, it was not enough to account for the large amount of prolactin produced.

Chapter 5 Secretion of Hybrid Proteins in Oocytes

INTRODUCTION

Translocation into the lumen of the RER represents the initial step in transport of secretory proteins to their final destination. The intracellular route of secretory proteins from the rough endoplasmic reticulum through the Golgi apparatus and to the cell surface is well established (1). Plasma membrane glycoproteins are also synthesized on RER, and this class of proteins is known to follow a similar route (72-76). A third class of proteins, the lysosomal enzymes are also initially synthesized on the RER, and this class of molecules is diverted from the secretory pathway at some point during its transit through the Golgi (77-78). The mechanisms by which these proteins are transported and sorted into different intracellular compartments are still poorly understood.

The best understood mechanism for sorting involves the mannose-6-phosphate recognition system for lysosomal enzymes. The newly synthesized enzymes receive high-mannose-type oligosaccharides in the RER, and they then move onto the Golgi complex where specific mannose residues of the oligosaccharides are phosphorylated (36-39). The processed oligosaccharide chains then bind to mannose-6-phosphate receptors which play a role in ensuring proper

delivery to the lysosomal compartment. Though proteins shuttled through the vesicular pathways receive a large number of modifications this is the only situation where a functional role for a particular modification has been identified.

Many membrane proteins and secretory proteins receive N-linked oligosaccharides, and there have been numerous attempts to assign a role for this modification in intracellullar transport. In particular, numerous investigators have utilized tunicamycin, an inhibitor of N-linked glycosylation, and compared rates of transport and ability to reach the cell surface (40, 80-81). These studies have yielded widely varying results, with the transport abilities of some molecules being greatly impaired while other molecules were seemingly unaffected. In many instances, glycosylation may have a critical influence on the conformation and solubility characteristics of the protein, and this may directly influence mobility through the intracellular membranous system.

Kinetic studies have shown that different secretory proteins are released into the medium at different rates, and that this difference is generally due to a difference

in the migration rate from the RER to the golgi (81-84). This has led to the hypothesis that there are membrane-bound receptors which selectively mediate transport, and which bind with different affinities to different ligands. As a corollary to this, it has been suggested that secretory proteins which are slowly transported might be transported by bulk-phase movement of the lumenal contents.

It was recently reported that bacterial beta-lactamase expressed in Xenopus oocytes was secreted into the medium (85). Beta-lactamase is a bacterial protein normally translocated into the periplasm. These authors suggested that a bacterial protein, which had not been adapted to the eukaryotic transport pathway, should not contain any of the sorting signals thought to interact with transport receptors in eukaryotic cells, and that it was therefore possible to traverse the secretory pathway without the need of a signal other than the leading signal sequence.

Since we had shown that the b-lactamase signal sequence was sufficient for translocation of globin in Xenopus oocytes, we wanted to know if this same globin molecule was capable of being secreted. To our surprise, we found that the oocytes synthesized a higher molecular

weight form of the globin molecule not normally seen in the in vitro translation system. This higher molecular weight protein was preferentially secreted. Other hybrid proteins containing varying parts of the amino terminus attatched to a globin molecule produced only one mature form of the protein, and none of these other proteins were capable of being secreted.

RESULTS

We have previously shown that the 125E molecule is capable of being translocated into oocyte vesicles and is processed. We were interested in knowing if this same molecule was capable of being secreted. Oocytes were injected with capped transcripts prepared from SP125E DNA, preincubated overnight, pulse-labelled in medium containing 5mC/ml S35-MET, then transferred to medium comtaining 20uM cold methionine, and incubated for various time intervals. After incubation, the oocytes and medium were separated, immunoprecipitated with globin antiserum, and analyzed by gel electrophoresis followed by autoradiography. Figure 11 shows the results of this experiment. Lanes A,C,E,G, and I show the products from the homogenates, and lanes B,D,F,H,J show the products in the medium; lanes A and B show the 1-hour pulse, and lanes C-D, E-F, G-H and I-J show the results of a 1, 3, 5, and 9-hour pulse respectively. In the 1-hour pulse, we see only one globin form which migrates to the position of the processed globin chain produced in the cell-free system. After a 1-hour chase, a second band of higher molecular-weight band appears, and after 9 hours of chase, most of the radioactively labelled material is seen in the upper molecular weight

band, almost all of which is in the medium. This data suggests that the upper molecular weight band is produced by post-translational processing of the lower molecular weight band first detected at 1 hour. Our calculations of the molecular weights of these two bands shows that the lower molecular weight band migrates to a position appropriate for a 14,000 dalton protein, and that the upper molecular-weight band migrates to the position of a 15,000 molecular-weight protein.

If this slower-migrating protein is being secreted by an exocytotic mechanism, then it should be contained within a vesicle population. Figure 12 shows the results of a subcellullar fractionation on oocytes labelled with S35-MET for periods of 4.5 hours. For these studies, the oocytes were homogenized in an iso-osmotic solution, and then separated into cytosolic and vesicle fractions after centrifugation through 20% sucrose. In this experiment, we prepared two sets of oocytes in which oocytes were injected with a mixture of transcripts. Lanes a-d show the results obtained from oocytes coinjected with transcripts coding for BP3 (preprolactin) and G1E (cytosolic globin). Lanes a and b show the cytosolic and vesicle fractions obtained with G1E. The G1E protein separated relatively evenly, with

a minor preference for the cytosolic fraction, however, prolactin expressed in the same set of cells appeared predominantly in the vesicle fraction. Lanes e-h show the results from oocytes injected with a mixture of 125E and BP3. In this case, both 125E and BP3 were contained predominantly in the membrane pellet. The even distribution of G1E most likely represents an ability of this molecule to be adsorbed to the vesicle surface; the following experiments in which proteins not sequestered inside vesicles are proteolyzed confirms that all of the G1E chains are on the outside.

If 125E is sequestered in vesicles, then it should be protected from protease digestion. Figure 13 shows the results of a proteolytic digestion with proteinase K on oocyte homogenates. Labelled oocytes were homogenized in an iso-osmotic buffer containing .25M sucrose, the nuclei and debris were removed by centrifugation in an eppendorf for 20 sec., and the resulting homogenate was then proteolyzed. In this experiment, we show oocytes coinjected with 125E (lanes a-c), and preprolactin (lanes d-f), or coinjected with G1E (lanes g-i) and BP3 (lanes j-l). Lanes a,d,g,j are the total products; lanes b,e,h,k show total products digested with protease; and lanes c,f,i,l show totla products digested with protease in the presence

of 1% triton. One can see that in the absence of detergent, 125E and BP3 are both protected, whereas G1E is completely digested. Proteolysis in the presence of Thus, both the proteins represented in oocytes injected with 125E are sequestered inside a vesicle population.

Previous studies of secretion in oocytes have shown that secretory proteins are transported at different rates (49) Figure 14 shows a pulse-chase experiment demonstrating that secretion of prolactin under the same conditions yields a time course similar to that of 125E with almost all of the prolactin being secreted within 17 hours. In this experiment. oocytes were injected with BP3 capped transcripts, pulse labelled for 3 hours with 5 mC/ml S35-met in MBSH (lanes A-B) and then chased for 7 (C-D) and 14 (E-F) hours in MBSH medium containing 20uM cold methionine. Lanes A,C,E are the oocyte homogenates, and lanes B,D,F are the medium. Thus both of these molecules are exported very efficiently compared with other proteins. Other experiments in our lab have shown that lactalbumin cannot be detected in the medium until a minimal incubation time of 50 hours has passed, and even then, a very small proportion of the total lactalbumin synthesized is in the medium at this point in time. Thus the modified globin

molecule is transported very efficiently.

Our results suggested that the more slowly migrating globin molecule is selectively transported; an alternative explanation is that both globin forms were secreted, however the lower molecular-weight form was selectively degraded in the medium. Figure 15 shows the results of an experiment in which we tested for this possibility by extracting injected oocytes and then adding the supernatant to the MBSH medium in the absence (lanes b-e) and presence (lanes f-i) of oocytes. Media were incubated for various time intervals (0,1,5,10 hours) and subsequently immmunoprecipitated and analyzed by gel electrophoresis. The results of these experiments show that though there was some degradation during the 10-hour time period tested, there was no selective degradation of the lower molecular-weight band. Thus, we can conclude that the upper molecular- weight band of globin was the only form capable of being secreted. Lanes j and k show medium incubated for 5 and 10 hours in the presence of 1000u/ml Trasolyl.

Our time course suggested that the slower migrating band was the result of a post-translational modification. We therefore checked to see if the appearance of this form was inhibited with monensin. Monensin is a carboxylic ionophore which inhibits secretion of most molecules,

usually by blocking transport in the mid to trans Golgi region (86). Thus, oocytes were injected with 125E and labelled for 10 hours with and without 10uM monensin added (Figure 16). In the presence of monensin (lanes a-b), only the lower molecular-weight band is seen at this 10-hour time point, and no globin products are detected in the medium. In the absence of monensin (c and d), both bands are present in equal amounts inside the cell, and some of the upper molecular-weight band has been secreted into the medium. These results suggest that the slower migrating band may be the results of a post-translational modification which takes place in the golgi. We are unable to make a strong statement about which stage of the transport might have been blocked by monensin because this is an inhibitor which can have different effects on different molecules or on different cell types. However, this experiment lends support to the idea that the upper molecular-weight band is the result of a post-translational modification.

In a series of earlier experiments we had begun to express a fusion protein coding for the beta-lactamse signal plus five additional amino acids fused to globin (GB14). Though this particular protein differed from 125E

only by the inclusion of 5 extra amino acids from the amino terminal coding region of beta-lactamase, expression of this hybrid protein produced only one detectable form of globin, and none of this protein was detected in the medium even after a 60-hour incubation. Figure 17 shows the results of a time course run on oocytes expressing GB14. Injected oocytes were labelled for 6 (A-D), 10 (E-F), 30 (H-I) and 60 (J-K) hours. AT the end of these time periods, medium (M) and oocytes (H) were separated, and the GB14 products were immunoprecipitated with anti-globin antiserum.

Two other constructions containing varying amounts of the amino terminus of secretory proteins fused to globin were also tested for their abilities to be secreted. One construction, pSPG2, contains the first 182 amino acids of beta-lactamase fused to globin, and the second construction, pSPBPG3 contains the first 86 amino acids of beta-lactamase fused to globin. Figure 18 shows that even after 72 hours, neither of these hybrid proteins were detected in the medium. Lanes C and D show the homogenate and medium of pSPG2, and lanes E and F show homogenate and medium from pSPBPG3. Thus, the transport of the globin domain appears to be an event which is fairly restricted.

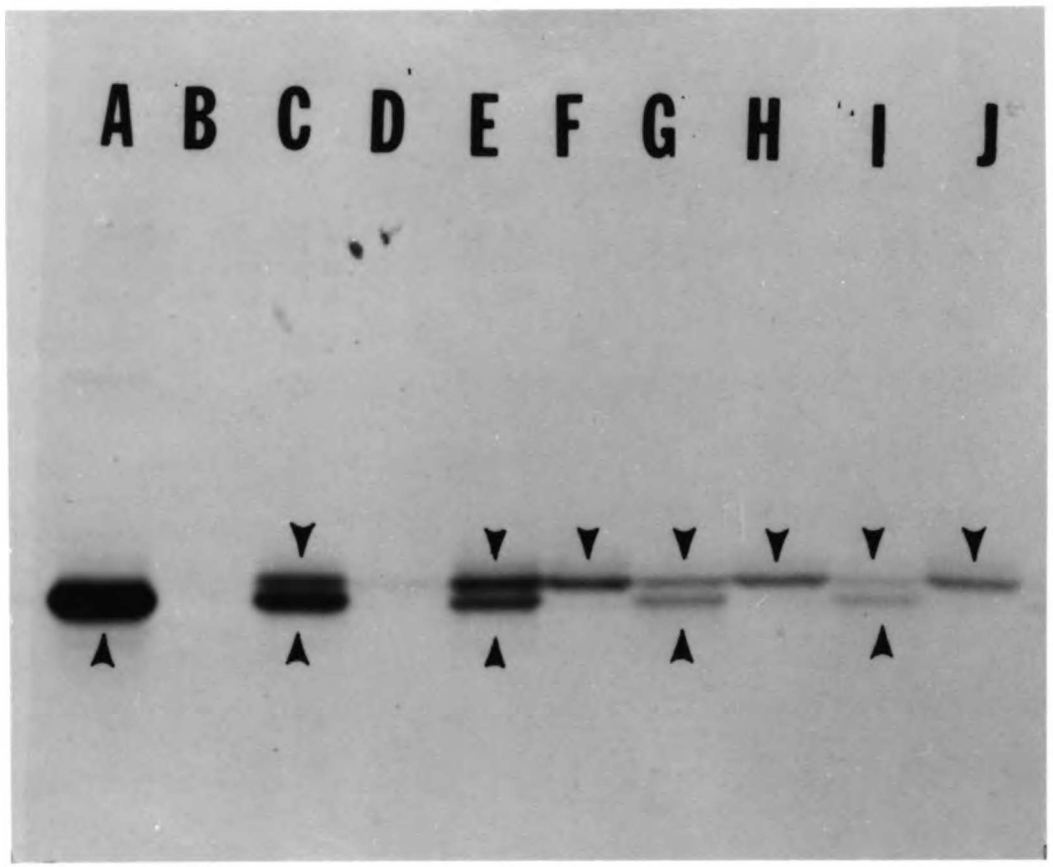

A B C D E F G H I J

fig.11 Time course of secretion of 125E expressed in Xenopus oocytes.

Oocytes were injected with 125E capped transcripts, preincubated for 24 hours in unlabelled medium and pulse-labelled for 1 hour in 5mc/ml S35-MET. After 1 hour, the hot medium was removed and replaced with cold chase medium consisting of 20mM MET, and groups of 3 oocytes were then incubated for another 1 (C-D), 3 (E-F), 5 (G-H), and 9 (U-J) -hour period. Lanes A and B show the 1-hour period. A,C,E,G show the products from homogenates, and B,D,F,G show products from the medium. S35-MET for time periods of 1.5, 3, 6, 14, and 26 hours. At the end of each incubation period, the medium (M) was separated from the oocytes (H), which were subsequently homogenized in buffer containing 1% Triton. Samples were analyzed by immunoprecipitation with anti-globin antiserum followed by gel electrophoresis followed by autoradiography. Upward-pointing arrowhead indicated position ofprocessed globin seen in the cell-free system. Downward-pointing arrowhead refers to novel globin form seen expressed only in oocytes.

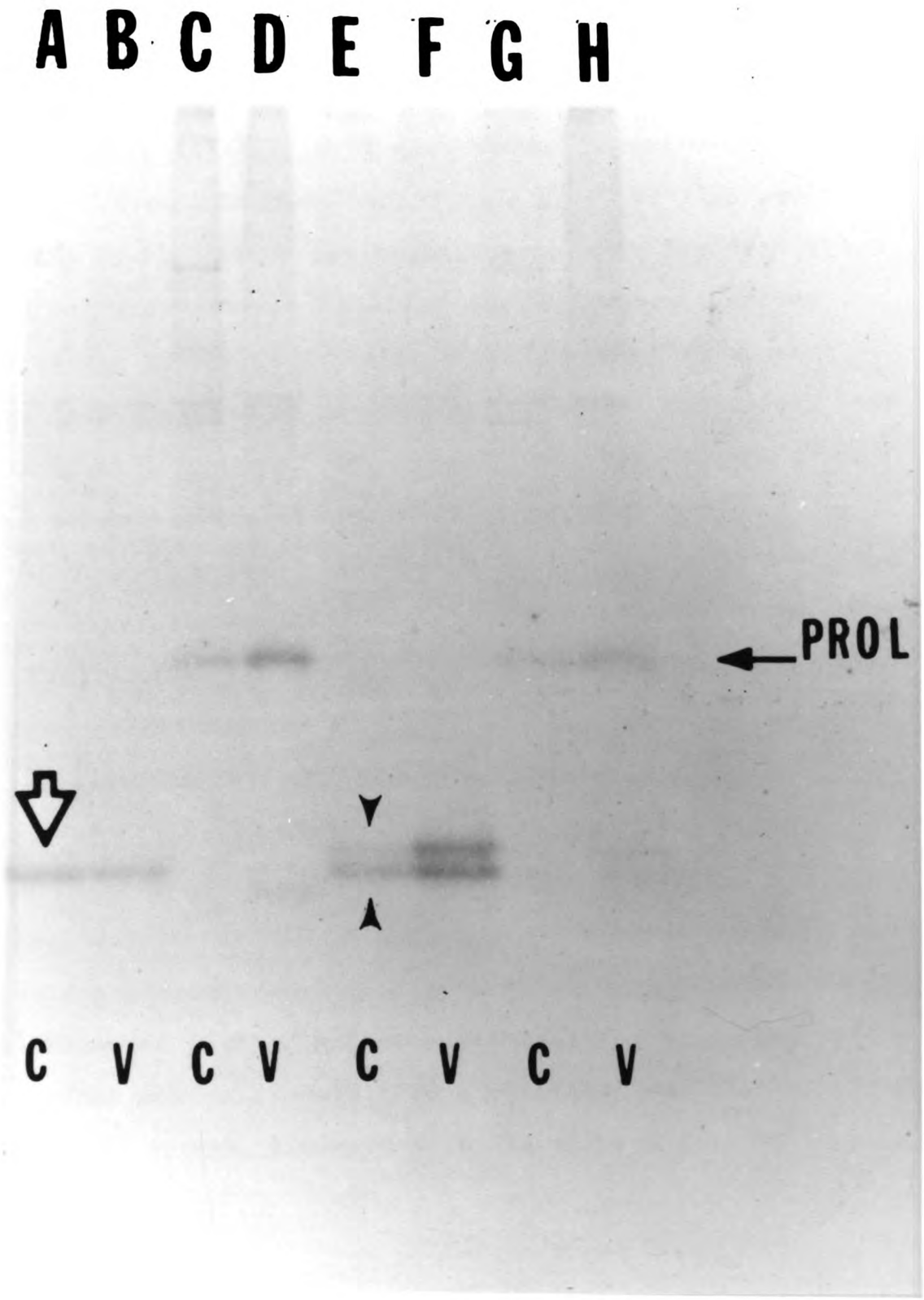

A B C D E F G H
PROL
C V C V C V C V

fig.12 Subcellular fractionation of injected oocytes.

Oocytes were injected with mixtures of capped transcripts prepared from G1E (A-B) and BP3 DNA (C-D) or 125E (E-F) and BP3 DNA (G-H). After preincubation in MBSH for 24 hours, injected oocytes were labelled for 4.5 hours in medium containing 5mC/ml of S35-MET. Freshly-labelled oocytes were then homogenized in iso-osmotic buffer containing lomM MgAc and 10% sucrose. Total homogenate was layered on top of 1 ml buffer containing 20% sucrose, and then centifuged in a swinging bucket rotor for 1/2 hour at 12,600 RPM. The upper layer representing the cytosol (c) fraction was collected, the 20% sucrose layer was discarded, and the pellet containing the membranes and vesicles (v) was resuspended in 1% Triton. All samples were then immunoprecipitated either with anti-prolactin antiserum (C,D,G,H), or with anti-globin antiserum (A,B,E,F). Downward-pointing and open arrowhead refers to cytosolic globin products from G1E. upward-pointing arrowhead refers to processed globin, and downward-pointing arrowhead refers to higher molecular-weight form of globin not observed in cell-free system. Sideward-pointing arrow refers to prolactin.

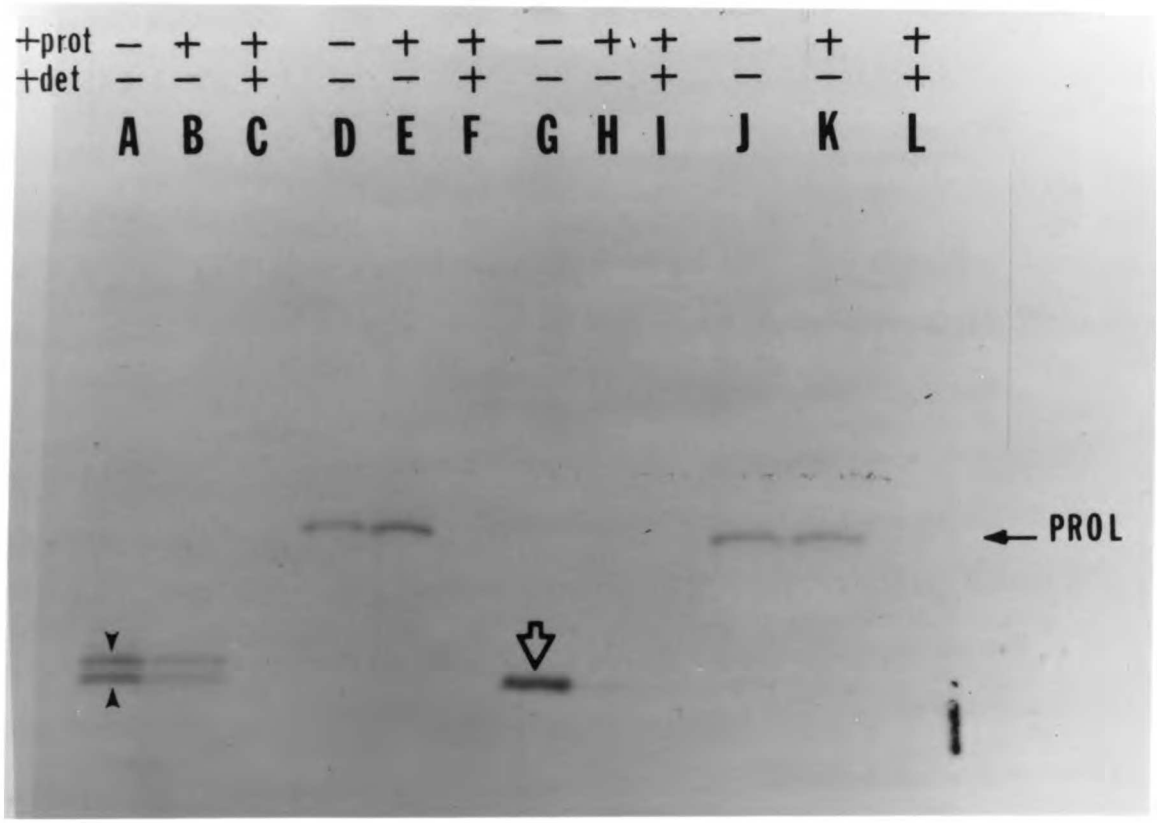

+prot – + + – + + – + + – + +
+det – – + – – + – – + – – +
A B C D E F G H I J K L
PROL

fig.13 Proteolysis of injected oocytes.

Oocytes were injected with mixtures of capped transcripts prepared from 125E and G1E DNA (A-F), and from G1E and BP3 DNA (G-L), preincubated in unlabelled meium for 24 hours, and subsequently labelled for 4.5 hours in MBSH containing 5 mC/ml S35-MET. Each set of injected oocytes was then divided into 3 aliquots, and one set was not further processed (A,D,G,J), a second set was digested in the presence of .3mg/ml Proteinase K for 3 hours at OC (B,E,H,K), and the third set was digested in .3mg/ml Proteinase K for 3 hours at OC in the presence of 1% Triton (C,F,I, L). Proteolysis was terminated by boiling in 4xvolume of 1% SDS for 10 minutes. Samples were analyzed by immunoprecipitation with anti-globin antiserum (A-C,G-I), or with anti-prolactin antiserum (D-F, J-L). Upward pointing arrowhead refers to globin which migrates to position of processed globin, upward-pointing arrowhead refers to higher molecular-weight form of globin seen only in oocytes, downward-pointing open arrow refers to cytosolic globin, and sideward-pointing arrow refers to prolactin.

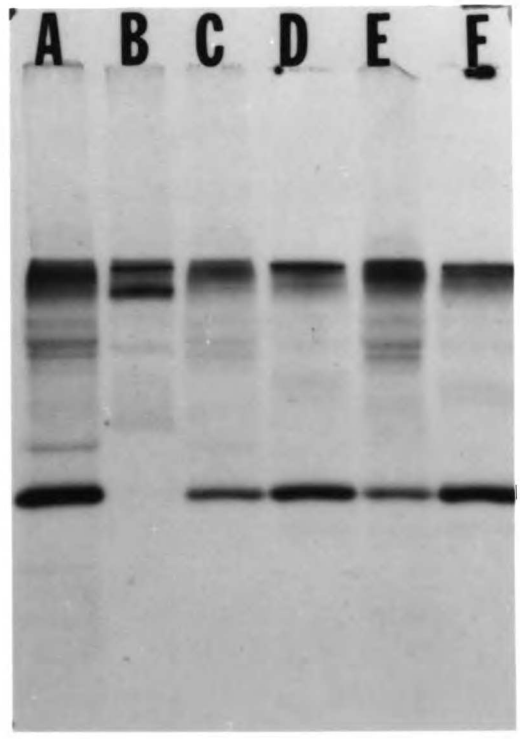
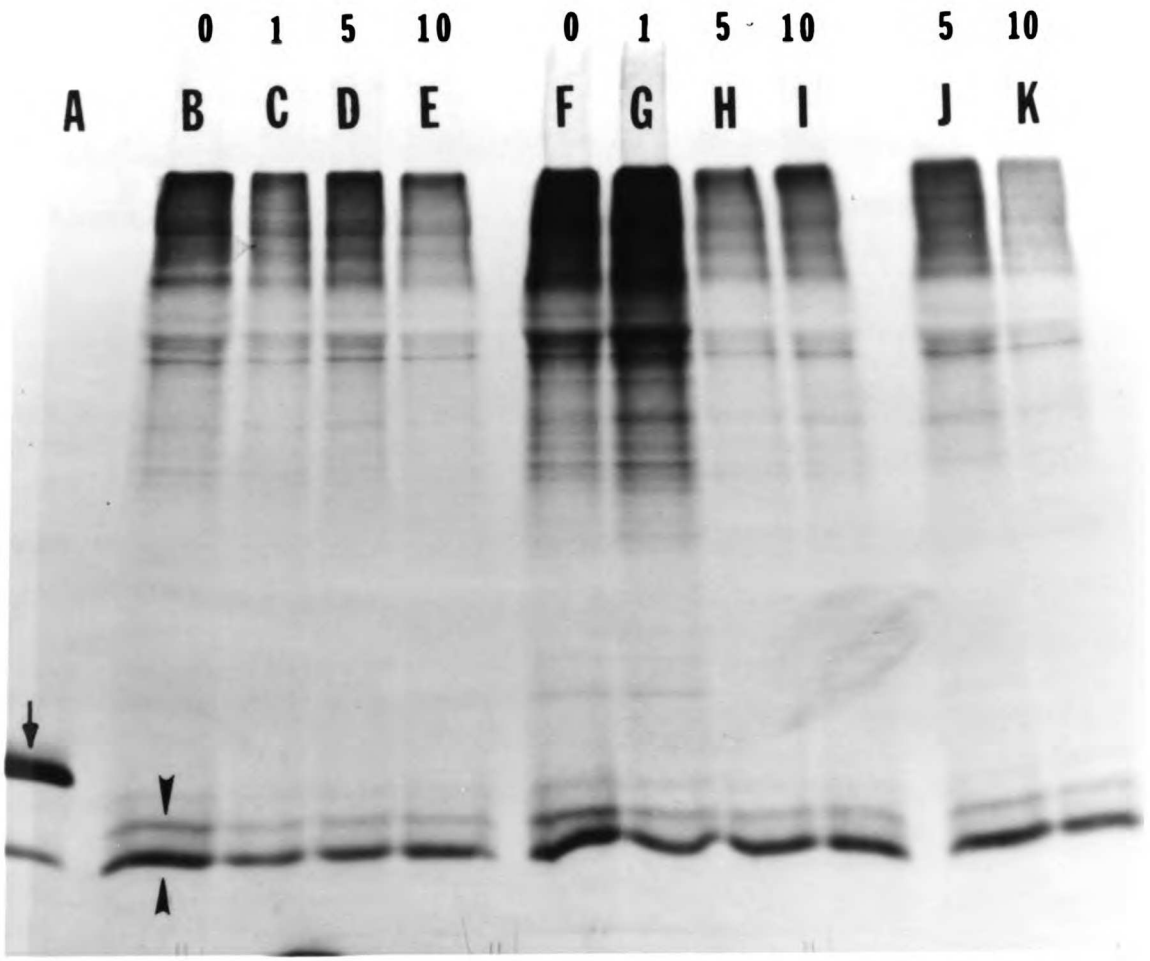

A B C D E F

fig.14 Time course on oocytes injected with BP3 capped transcripts.

Oocytes were injected with BP3 capped transcripts, preincubated for 24 hours in unlabelled MBSH, pulse-labelled for 3 hours (A-B) in MBSH containing 5mc/ml S35 MET, and then chased for 7 (C-D), or 14 (E-F) hours in MBSH with 20mM cold methionine. At the end of the indicated time periods, the medium was separated from oocytes which were homogenized in 20ul/oocyte of buffer containing 1% Triton. Samples were analyzed by immunoprecipitation followed by gel electrophoresis and autoradiography. Lanes A,C,E are oocyte homogenates , and lanes B,D,F are oocyte medium.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K

fig.15 Time course for degradation of 125E oocyte products.

Oocytes were injected with 125E capped transcripts, labelled for 24 hours in medium containing 1mC/ml S35-MET, homogenized in buffer without Triton, and centrifuged in the airfuge at 100,000g for 1 hour. Supernatants were collected, and then diluted 1/10 in MBSH medium which was subsequently incubated at 19C in the absence (B-E) and in the presence (F-K) of uninjected oocytes. Lanes J and K show medium to which 1000u/ml of trasolyl were added. Lane A shows the translation products synthesized in the cell-free system. Downward-pointing arrow indicates precursor, upward-pointing arrowhead refers to processed globin, and downward-pointing arrowhead refers to higher molecular-weight form of globin seen only in oocytes. Time periods were 0 (B,F), 1 (C,G), 5 (D,H,J) or 10 (E,I,K) hours.

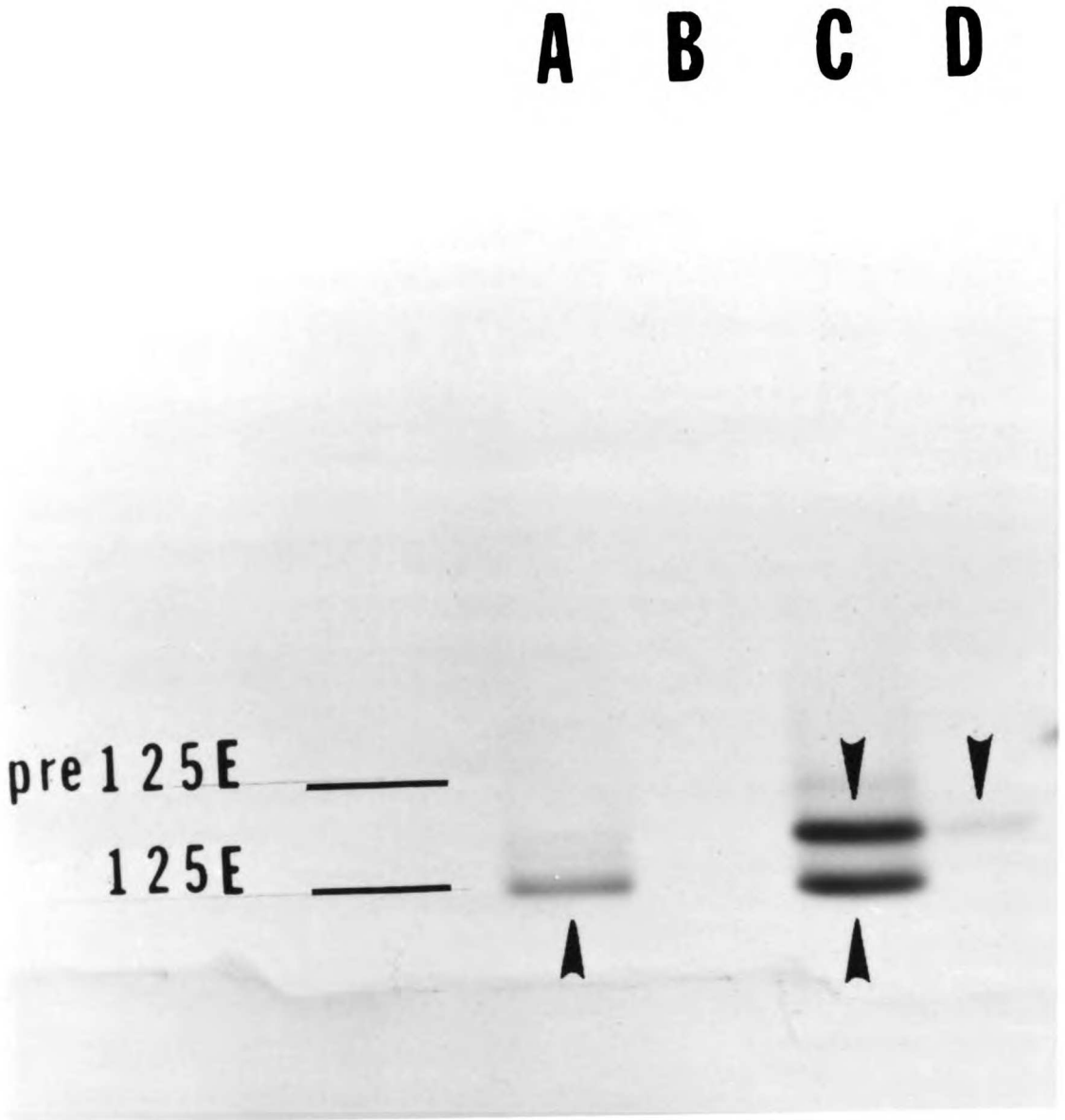
A B C D
pre 1 2 5E
1 2 5E

fig.16 Effects of monensin on 125E expression in oocytes.

Oocytes were injected with 125E capped transcripts and labelled for 10 hours in the presence (A,B) and absence (C,D) of 10uM monensin. Following the incubation period, the medium (m) was harvested and oocytes (h) were homogenized in buffer containing 1% Triton. Upward-pointing arrowhead refers to processed globin, and downward-pointing arrowhead refers to modified form of globin.

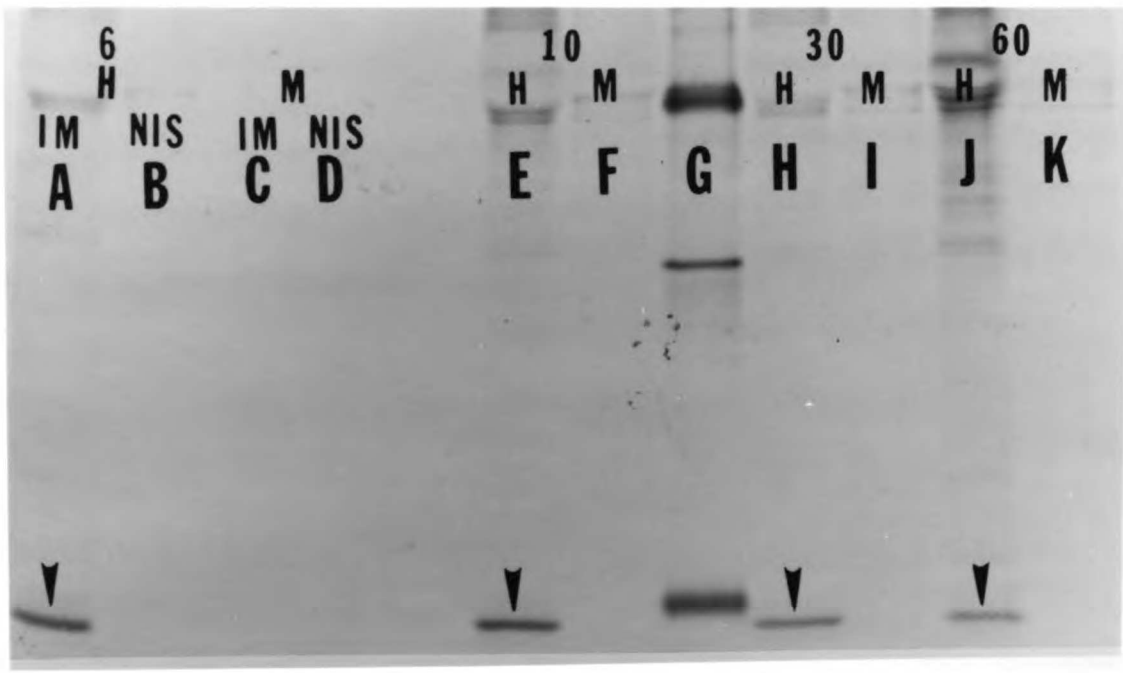
6
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fig.17 Time course on GB14 expressed in oocytes

Capped transcripts were prepared from GB14 DNA and injected into oocytes. Oocytes were incubated for varying time periods in MBSH containing 1mC/ml S35-MET. At the end of the incubation period, the medium (m) was harvested, and the oocytes (h) were homogenized in buffer containing 10% Triton. Samples were analyzed by immunoprecipitation followed by gel electrophoresis. Lanes A-D show injected oocytes which were incubated in labelled medium for 6 hours and then immunoprecipitated with immune (A,C) or non-immune (B,D) serum. Lanes E, H, J show the homogenates from oocytes labelled for 10, 30, and 60 hours respectively. Lanes F, I, K show the medium from oocytes incubated for 10, 30, and 60 hours respectively. Lane G is the molecular weight markers.

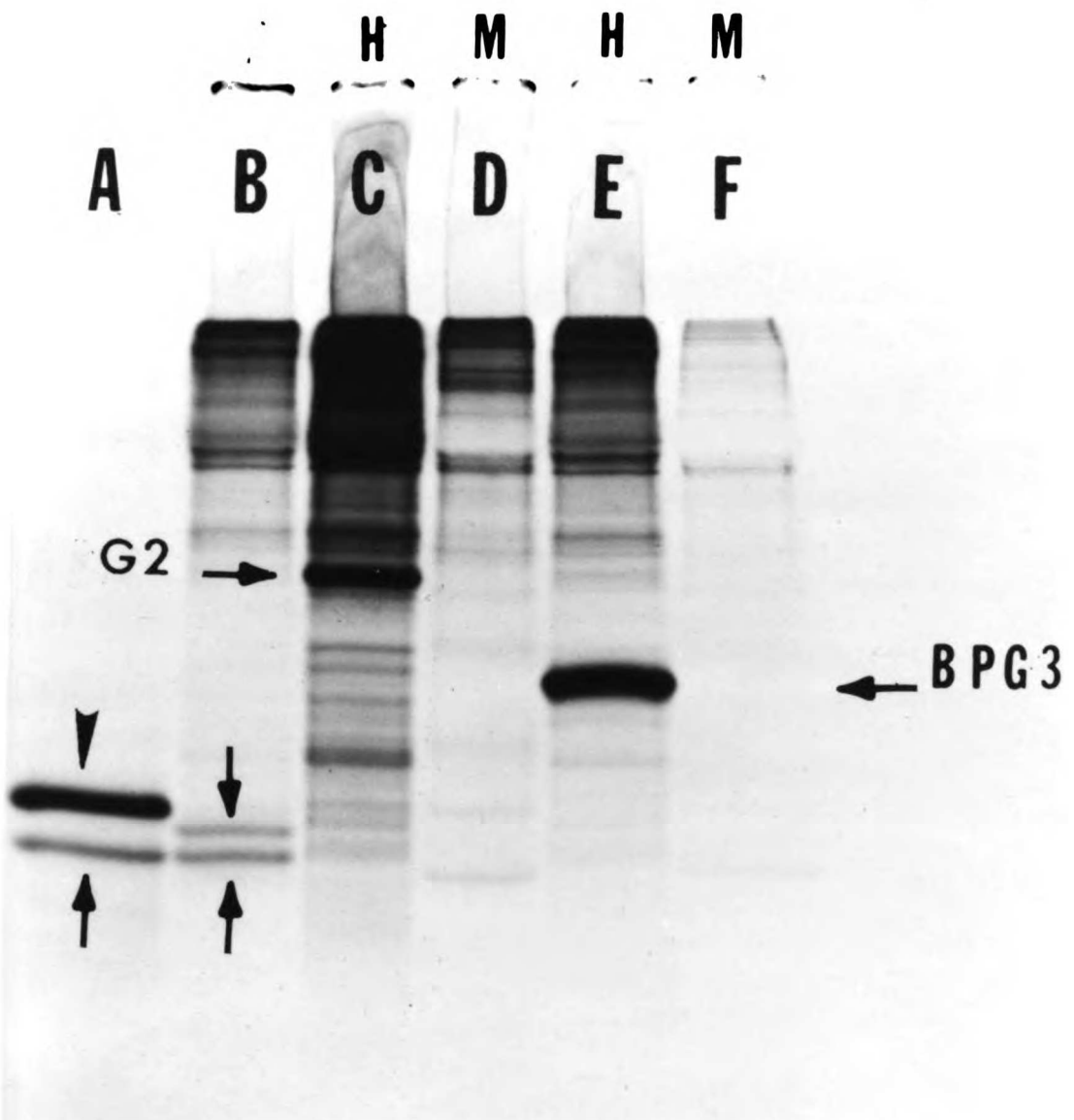
H
M
H
M
A
B
C
D
E
F
G2
BPG3

fig.18 Expression of SPG2 and SPBPG3 in oocytes.

Oocytes were injected with capped transcripts and labelled for 72 hours in MBSH containing 1mC/ml S35-MET. At the end of the incubation period, the medium (M) was harvested and the oocytes (H) were homogenized in buffer containing 1% Triton. Downward pointing arrowhead refers to 125E precursor seen in cell-free translation, upward-pointing arrowhead refers to processed globin, and downward-pointing arrowhead refers to modified globin. Sideward-pointing arrows refer to SPG2 and SPBP3.

CONCLUSIONS

We have shown that expression of the 125E hybrid protein in Xenopus oocytes produces two forms of the globin molecule, and both forms are processed and sequestered inside vesicles. One form of the protein migrates to the same position as the processed globin chain which we observe in the cell-free system, and a second form migrates with a slightly slower mobility. Our results strongly suggest that the higher molecular-weight form of globin results from a post-translational modification of the lower molecular-weight form. This interpretation of our results is supported by three different lines of evidence: firstly, pulse-chase studies uielded only the lower molecular-weight peptide after a one-hour pulse. Secondly, the higher molecular-weight form expressed in oocytes is not observed in the cell-free system, even in the presence of dog pancreas microsomes. This finding suggests that what we observe might be the result of a processing step which occurs in a stage of the transport pathway later than the ER. The third line of evidence is from experiments in which incubation of injected oocytes in medium containing 10uM monensin completely blocked conversion of globin molecules to the higher molecular-

weight form and completely abolished secretion. The third line of evidence comes from pulse-chase studies in which a one-hour pulse yielded only the lower molecular-weight band. We are presently sequencing these two proteins to reconfirm our interpretation.

Attempts to identify this modification have so far been unsuccessful, though we have succeeded in eliminating a number of potential candidates. N-linked glycosylation is a highly unlikely explanation. It is well-established that N-linked oligosacchardies are attatched to an asparagine residue within the tripeptide sequence ASN-X-SER/THR; no such sequence exists in the coding region for the chimpanzee globin molecule which we have fused to the b-lactamase signal. N-linked oligosaccharides are attatched co-translationally in the endoplasmic reticulum, and the membranes we used in our in vitro translation system are capable of adding this modification. Additionally, the inclusion of 2uM tunicamycin in the oocyte incubation medium did not inhibit the appearance of the modifidied globin form, though glycosylation was inhibited in controls. Thus, the modification we observed is probably not due to N-linked glycosylation.

Phosphorylation of the globin molecule might cause the shift in migration mobility detected in our SDS gels.

Experiments in which oocytes were injected with 125E transcripts and then incubated for 10 hours in medium which included 1mC/ml of P32-orthophosphoate yielded negative results. Control oocytes incubated in S35-methionine showed that the transcripts were properly translated and that the modified 125E peptide was expressed. One very strong candidate to explain our results would be the addition of oligosaccharide units linked O-glycosidically to a serine or threonine residue. There is an increasing body of evidence suggesting that the initial step in O-linked glycosylation occurs post-translationally in the smooth ER or the Golgi apparatus (96-98). We are presently testing for this possibility.

Our pulse-chase experiments demonstrate that the modified globin molecule synthesized in oocytes is very efficiently and rapidly secreted. By 17 hours, greater than 90% of the globin molecules are in the extracellular medium; this is a time course similar to that observed for prolactin, a protein which is normally secreted from anterior pituitary cells. The pulse-chase also reveals that once the globin molecule has been converted, modified globin molecules are very rapidly secreted.

Kinetic studies also reveal that during the 17-hour

hour chase period, there is a decrease in the total amount of immunoprecipitable globin chains. Though we do not have conclusive evidence, the decrease in the amounts of globin seen in the medium over the 17-hour time period suggests that at least some of the secreted molecules were degraded in the medium, possibly by the proteases known to be secreted by oocytes and their surrounding follicle cells (55). 10% FCS was included in the medium in order to minimize degradation by extracellular proteases. Earlier experiments in which pulse-chase studies were conducted in the absence of FCS yielded no detectable globin or prolactin either intracellularly or in the medium after a 10-hour chase. We assume that the inclusion of fetal calf serum has lessened the effects of extracellular degradation, though we cannot exclude the possibility that the inclusion of fetal calf serum has somehow stimulated the secretion of both prolactin and 125E.

The finding that the globin molecule is secreted, and that its transport was so rapid and efficient is intriguing. Globin is a cytosolic protein which normally would never enter the vesicular pathway. This, in fact, is the first report of a protein which is not normally

targetted to the ER entering the secretory pathway. Other investigators have altered the genes coding for transmembrane proteins by deleting cDNA sequences coding for the transmembrane domain and the cytosolic tail on the carboxy terminus (4-44,47). Expression of truncated forms of VSV G protein and influenza hemagglutinin (HA) in cultured cells led to the slow secretion of both glycorproteins. The slow transport of truncated HA and G proteins could have been caused either by the loss of a sorting signal which normally guides these proteins into transport vesicles or by the altered proteins taking on a conformation which reduces solubility and thereby impedes transport.

In the above studies, proteins which normally contain all of the information necessary for efficient transport to the cell surface were altered. By contrast, we have introduced into the lumen of the ER an unaltered protein which in its natural locatio has no requirement for any of the putative sorting or transport signals. The efficiency with which this native molecule is exported suggests that the slowed or blocked transport of altered proteins may be due to their inability to assume their native conformation. It is significant that a fusion protein coding for the b-lactamase signal plus 5 additional amino

acids of b-lactamase fused to globin (GB14) was not able to be secreted, even after very long time periods. Thus, the addition of just 5 amino acids was sufficient to completely block secretion of globin chains. Other investigators working with membrane proteins found that addition of novel sequences to the cytosolic tail at the carboxy terminus of membrane glycoproteins inhibited transport. In the present study, we included a very small domain of a naturally-secreted protein (b-lactamase) and we added this sequence to the amino terminus of a secreted protein (125E). Our results show that very small changes in the primary amino acid sequence can lead to significant changes in transport behavior of secreted proteins. A similar result was reported by Wu et al (89), who found that a single amino acid substitution in the variable region of an immunoglobulin light chain completely blocked its secretion. It is thus clear that there are severe restraints on the context within which a protein domain must reside in order to be efficiently transported. We have also shown that in 3 different fusion proteins, addition of varying amounts of the amino terminus of a secretory protein (b-lactamase) fused to the coding region for globin inhibited the ability of this molecule to be

exported.

It is noteworthy that none of the fusion proteins which contained b-lactamase coding sequences fused to globin yielded a second higher molecular-weight form of immunoprecipitable globin material. Apparently, the same conformational changes which resulted in impaired transport ability also inhibited addition of the post-translational modification. Likewise, incubation of oocytes in monensin inhibited both secretion and conversion to the higher molecular-weight form. It is apparent that there is a very strong correlation between the ability of globin to be secreted and the acquisition of this modification. In no case did we observe a protein which was modified but not secreted, or vice versa. There are two possible explanations for this behavior. The first is that the added modification serves as a sorting signal which specifically recognizes a receptor on transport vesicles; in this case, slight alterations in conformation would cause the critical domain in the fusion protein to become inaccessible to enzymes which attatche the necessary sorting signal. Alternatively, the additional amino acids in GB14 may have caused globin to assume an unfavorable conformation which directly impedes transport, and that GB14 was therefore unable to arrive at

the critical compartment where it would receive the modification; in this case, we would assume that addition of the modification is a fortuitous event which provides no additional information necessary for export.

Previous studies have demonstrated that in the same cell different proteins (both secretory and membrane-bound) are tranaported to the cell surface at characteristically different rates (81-84, 88), and that these varying rates can be accounted for by differences in rates of migration from the ER to the Golgi. On the basis of this evidence, these authors have proposed that intracellular transport is a selective rather than a passive flow process which involves the binding of these proteins to a membrane-bound receptor which selectively mediates transport from the endoplasmic reticulum to the Golgi apparatus. These authors suggested that the binding affinity of the transported proteins would determine their rate of transfer; slow-moving proteins might not bind to the receptor and would be transported by bulk-phase movement of the lumenal contents. An alternative model is that alterations in exported proteins may lead to changes in conformation which could act directly to retard the movement of the molecules, possibly by impeding their

ability to be included in budding vesicles. Our results could be explained by either of the two models. Identification of the modification and a further characterization of its role in secretion is required to distinguish between the two possibilities. However, our results show that preservation of the exact primary amino acid sequence of the native protein is critical for efficient transport of a secreted protein.

DISCUSSION

It is well-established that secreted proteins contain at their amino terminus a signal sequence which is essential for their targetting to and translocation across the ER membrane (4-11). It has been postulated that other "topogenic sequences" reside within protein domains (95).A number of investigators have recently expressed hybrid proteins in cell-free systems and thus provided evidence for the existence of postulated "stop-transfer" and "internal" signals (87, 93, 94).

The results presented in chapter 4 demonstrate that it is possible to artificially construct hybrid proteins which are capable of utilizing the predesignated signal sequences both in vivo and in vitro. We have demonstrated that, except for minor differences, the continued use of the cell-free system to characterize these artificially engineered proteins yields a faithful presentation of translation events.

In the present studies we utilized proteins with reasonably simple orientations, however, it is conceivable that molecules with a more complicated topology may in fact behave differently in the two systems. It is also possible that some molecules may have an initial topological orientation which changes with time. Recent evidence from cell-free systems suggests that Hepatitis B Virus is

initially synthesized as an integral membrane protein, though it has been detected in cell culture only as a secreted particle for (98-99). Whereas the in vitro system functions for a period of only one hour, the microinjection system would allow one to perform a kinetic study on a protein whose topographic orientation is in flux.

Our results with the GP fusion protein, in which a previously amino terminal signal has now been placed in an artificial location, suggest that amino terminal and internal signals are functionally related. Since the internally placed signal is used less efficiently, there may be either constraints on, or added requirments for, the efficient use of an internally located signal.

The use of the microinjection system has enabled us to ask about the ability of hybrid proteins to be transported further along the secretory pathway. We find that the previously cytosoloic globin domain is efficiently and rapidly secreted, but only if it is introduced with its prinary amino acid sequence unaltered. Addition of as few as 5 amino acids from mature b-lactamase completely inhibited secretion. These findings are in agreement with the growing body of literature demonstrating that minor alterations in primary amino acids sequence of normally transported proteins can greatly inhibit their transport abilities. These studies have shown that additions, and mixed combinations of chimaeric proteins may poison transport ability, despite the fact

that none of the putative "sorting signals" have been removed. It is possible that minor alterations in primary amino acid sequence may induce conformational changes which greatly effect protein-protein interactions and abilities of molecules to be vesicularized. Therefore, the quest for sorting domains is complicated by the difficulties in determining or predicting tertiary structure.

The fact that the 125E globin domain has received a modification en route is intriguing. Identification and localization of this modificiation within the polypeptide chains should allow us to characterize its role in transport. This may be a modification which is commonly added in oocytes and not in other cells. At present, we have almost no knowledge of the various transport pathways and processing steps utilized by oocytes. Preliminary evidence suggests that in fact the composition of subcellular components differs from that of other cells (100).

Since oocytes are very large, it is conceivably possible to inject these cells with cytosolic components or with vesicle populations suspected to play a critical role in transport events. It is thus possible to manipulate the intracellular as well as the extracellular environment. The present work outlines the procedures for obtaining high-level expression of cloned genes and for performing a number of critical assays. We have demonstrated

that it is possible to coinject various ligands, and also to perform sequential injections on a single cell. We have only begun to explore the possible ways in which to manipulate the intracellular environment of oocytes.

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